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(71) Applicant (for all designated States except US): DE-GUSSA AG [DE/DE]; Bennigsenplatz 1, 40474 Düsseldorf (DE).

(72) Inventors; and

- (75) Inventors/Applicants (for US only): BATHE,
  Brigitte [DE/DE]; Twieten 1, 33154 Salzkotten (DE).
  KREUTZER, Caroline [DE/DE]; Poststrasse 16, 49326
  Melle (DE). MÖCKEL, Bettina [DE/DE]; Benrodestrasse 35, 40597 Düsseldorf (DE). THIERBACH, Georg [DE/DE]; Gunststrasse 21, 33613 Bielefeld (DE).
- (74) Common Representative: DEGUSSA AG; Intellectual Property Management, Patents and Trademarks, Location Hanau, P. O. Box 13 45, 63403 Hanau (DE).
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(54) Title: CORYNEFORM BACTERIA WHICH PRODUCE CHEMICAL COMPOUNDS II

(57) Abstract: The invention relates to coryneform bacteria, which instead of the singular copy of an open reading frame (ORF), gene or allele naturally present at the particular desired site (locus), have at least two copies of the open reading frame (ORF), gene or allele in question, preferably in tandem arrangement, and optionally at least a third copy of the open reading frame (ORF), gene or allele in question at a further gene site, and processes for the preparation of chemical compounds by fermentation of these bacteria.



### Coryneform Bacteria which Produce Chemical Compounds II

#### Prior Art

Chemical compounds, which means, in particular, L-amino acids, vitamins, nucleosides and nucleotides and D-amino acids, are used in human medicine, in the pharmaceuticals industry, in cosmetics, in the foodstuffs industry and in animal nutrition.

Numerous of these compounds are prepared by fermentation from strains of coryneform bacteria, in particular

10 Corynebacterium glutamicum. Because of their great importance, work is constantly being undertaken to improve the preparation processes. Improvements to the process can relate to fermentation measures, such as, for example, stirring and supply of oxygen, or the composition of the nutrient media, such as, for example, the sugar concentration during the fermentation, or the working up to the product form by, for example, ion exchange chromatography, or the intrinsic output properties of the microorganism itself.

20 Methods of mutagenesis, selection and mutant selection are used to improve the output properties of these microorganisms. Strains which are resistant to antimetabolites or are auxotrophic for metabolites of regulatory importance and which produce the particular compounds are obtained in this manner.

Methods of the recombinant DNA technique have also been employed for some years for improving the strain of Corynebacterium strains, by amplifying individual biosynthesis genes and investigating the effect on production.

A common method comprises amplification of certain biosynthesis genes in the particular microorganism by means of episomally replicating plasmids. This procedure has the disadvantage that during the fermentation, which in industrial processes is in general associated with numerous generations, the plasmids are lost spontaneously (segregational instability).

Another method comprises duplicating certain biosynthesis genes by means of plasmids which do not replicate in the particular microorganism. In this method, the plasmid, including the cloned biosynthesis gene, is integrated into the chromosomal biosynthesis gene of the microorganism 10 (Reinscheid et al., Applied and Environmental Microbiology 60(1), 126-132 (1994); Jetten et al., Applied Microbiology and Biotechnology 43(1):76-82 (1995)). A disadvantage of this method is that the nucleotide sequences of the plasmid and of the antibiotic resistance gene necessary for the 15 selection remain in the microorganism. This is a disadvantage, for example, for the disposal and utilization of the biomass. Moreover, the expert expects such strains to be unstable as a result of disintegration by "Campbell

Object of the Invention

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The inventors had the object of providing new measures for improved fermentative preparation of chemical compounds using coryneform bacteria.

type cross over" in a corresponding number of generations

such as are usual in industrial fermentations.

25 Summary of the Invention

The invention provides coryneform bacteria, in particular of the genus Corynebacterium, which produce one or more desired chemical compounds, characterized in that

a) instead of the singular copy of an open reading frame (ORF), gene or allele naturally present at the particular desired site (locus), these have at least two copies of the said open reading frame (ORF), gene or allele, preferably in tandem arrangement, no

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nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the particular site, and in that these

- b) optionally have at least a third copy of the open reading frame (ORF), gene or allele in question at a further gene site, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the further gene site.
- The invention also provides processes for the preparation of one or more chemical compounds, which comprise the following steps:
  - a) fermentation of coryneform bacteria, in particular of the genus Corynebacterium, which
- 20 instead of the singular copy of an open reading i) frame (ORF), gene or allele naturally present at the particular desired site (locus), have at least two copies of the said open reading frame (ORF), gene or allele, preferably in tandem 25 arrangement, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the particular site, 30 and in that these
  - ii) optionally have at least a third copy of the said open reading frame (ORF), gene or allele at a

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further gene site, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the further gene site,

under conditions which allow expression of the said open reading frames (ORFs) genes or alleles,

- b) concentration of the chemical compound(s) in the fermentation broth and/or in the cells of the bacteria,
  - c) isolation of the chemical compound(s), optionally
- d) with constituents from the fermentation broth and/or the biomass to the extent of > (greater than) 0 to 100%.

## Detailed Description of the Invention

Chemical compounds are to be understood, in particular, as meaning amino acids, vitamins, nucleosides and nucleotides.

The biosynthesis pathways of these compounds are known and are available in the prior art.

Amino acids mean, preferably, L-amino acids, in particular the proteinogenic L-amino acids, chosen from the group consisting of L-aspartic acid, L-asparagine, L-threonine, L-serine, L-glutamic acid, L-glutamine, glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan, L-proline and L-arginine and salts thereof, in particular L-lysine, L-methionine and L-threonine. L-Lysine is very particularly preferred.

Proteinogenic amino acids are understood as meaning the amino acids which occur in natural proteins, that is to say in proteins of microorganisms, plants, animals and humans.

Vitamins mean, in particular, vitamin B1 (thiamine), vitamin B2 (riboflavin), vitamin B5 (pantothenic acid), vitamin B6 (pyridoxines), vitamin B12 (cyanocobalamin), nicotinic acid/nicotinamide, vitamin M (folic acid) and vitamin E (tocopherol) and salts thereof, pantothenic acid being preferred.

10 Nucleosides and nucleotides mean, inter alia, S-adenosylmethionine, inosine-5'-monophosphoric acid and guanosine-5'-monophosphoric acid and salts thereof.

The coryneform bacteria are, in particular, those of the genus Corynebacterium. Of the genus Corynebacterium, the species Corynebacterium glutamicum, Corynebacterium ammoniagenes and Corynebacterium thermoaminogenes are preferred. Information on the taxonomic classification of strains of this group of bacteria is to be found, inter alia, in Kämpfer and Kroppenstedt (Canadian Journal of 20 Microbiology 42, 989-1005 (1996)) and in US-A-5,250,434.

Suitable strains of the species Corynebacterium glutamicum (C. glutamicum) are, in particular, the known wild-type strains

Corynebacterium glutamicum ATCC13032 25 Corynebacterium acetoglutamicum ATCC15806 Corynebacterium acetoacidophilum ATCC13870 Corynebacterium lilium ATCC15990 Corynebacterium melassecola ATCC17965 Corynebacterium herculis ATCC13868 30 Arthrobacter sp ATCC243 Brevibacterium chang-fua ATCC14017 Brevibacterium flavum ATCC14067 Brevibacterium lactofermentum ATCC13869

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Brevibacterium divaricatum ATCC14020 Brevibacterium taipei ATCC13744 and Microbacterium ammoniaphilum ATCC21645

and mutants or strains, such as are known from the prior art, produced therefrom which produce chemical compounds.

Suitable strains of the species Corynebacterium ammoniagenes (C. ammoniagenes) are, in particular, the known wild-type strains

Brevibacterium ammoniagenes ATCC6871
Brevibacterium ammoniagenes ATCC15137 and
Corynebacterium sp. ATCC21084

and mutants or strains, such as are known from the prior art, produced therefrom which produce chemical compounds.

Suitable strains of the species Corynebacterium

thermoaminogenes (C. thermoaminogenes) are, in particular, the known wild-type strains

Corynebacterium thermoaminogenes FERM BP-1539
Corynebacterium thermoaminogenes FERM BP-1540
Corynebacterium thermoaminogenes FERM BP-1541 and
Corynebacterium thermoaminogenes FERM BP-1542

and mutants or strains, such as are known from the prior art, produced therefrom which produce chemical compounds.

Strains with the designation "ATCC" can be obtained from the American Type Culture Collection (Manassas, VA, USA). Strains with the designation "FERM" can be obtained from the National Institute of Advanced Industrial Science and Technology (AIST Tsukuba Central 6, 1-1-1 Higashi, Tsukuba Ibaraki, Japan). The strains of Corynebacterium thermoaminogenes mentioned (FERM BP-1539, FERM BP-1540, FERM BP-1541 and FERM BP-1542) are described in US-A 5,250,434.

Open reading frame (ORF) describes a section of a nucleotide sequence which codes or can code for a protein or polypeptide or ribonucleic acid to which no function can be assigned according to the prior art.

After assignment of a function to the nucleotide sequence section in question, it is in general referred to as a gene.

Alleles are in general understood as meaning alternative forms of a given gene. The forms are distinguished by differences in the nucleotide sequence.

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In the context of the present invention, endogenous, that is to say species-characteristic, open reading frames, genes or alleles are preferably used. These are understood as meaning the open reading frames, genes or alleles or nucleotide sequences thereof present in the population of a species, such as, for example, Corynebacterium glutamicum.

A "singular copy of an open reading frame (ORF), gene or allele naturally present at the particular desired site (locus)" is understood as meaning the circumstances that a gene in general naturally occurs in one (1) copy in the form of its nucleotide sequence at its site or gene site in the corresponding wild-type or corresponding parent organism or starting organism. This site is preferably in the chromosome.

Thus, for example, the lysC gene or an lysC<sup>FBR</sup> allele which codes for a "feed back" resistant aspartate kinase is present in one copy at the lysC site or lysC locus or lysC gene site and is flanked by the open reading frame orfX and the leuA gene on one side and by the asd gene on the other side.

"Feed back" resistant aspartokinases are understood as meaning aspartokinases which, compared with the wild-type form, have a lower sensitivity to inhibition by mixtures of

lysine and threonine or mixtures of AEC (aminoethylcysteine) and threonine or lysine by itself or AEC by itself. Strains which produce L-lysine typically contain such "feed back" resistant or desensitized aspartokinases.

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The nucleotide sequence of the chromosome of
Corynebacterium glutamicum is known and can be found in the
patent application EP-A-1108790 and Access Number
(Accession No.) AX114121 of the nucleotide sequence

10 databank of the European Molecular Biologies Laboratories
(EMBL, Heidelberg, Germany and Cambridge, UK). The
nucleotide sequences of orfX, the leuA gene and the asd
gene have the Access Numbers AX120364 (orfX), AX123517
(leuA) and AX123519 (asd).

- 15 Further databanks, such as, for example, that of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) or that of the Swiss Institute of Bioinformatics (Swissprot, Geneva, Switzerland) or that of the Protein Information Resource Database (PIR, Washington, DC, USA) can also be used.
  - "Tandem arrangement" of two or more copies of an open reading frame (ORF), gene or allele is referred to if these are arranged in a row directly adjacent in the same orientation.
- "A further gene site" is understood as meaning a second gene site, the nucleotide sequence of which is different from the sequence of the ORF, gene or allele which has been at least duplicated at the natural site. This further gene site, or the nucleotide sequence present at the further gene site, is preferably in the chromosome and is in general not essential for growth and for production of the desired chemical compounds.

The "further gene sites" mentioned include, of course, not only the coding regions of the open reading frames or genes mentioned, but also the regions or nucleotide sequences lying upstream which are responsible for expression and regulation, such as, for example, ribosome binding sites, promoters, binding sites for regulatory proteins, binding sites for regulatory ribonucleic acids and attenuators.

These regions in general lie in a range of 1-800, 1-600, 1-400, 1-200, 1-100 or 1-50 nucleotides upstream of the coding region. In the same way, regions lying downstream, such as, for example, transcription terminators, are also included. These regions in general lie in a range of 1-400, 1-200, 1-100, 1-50 or 1-25 nucleotides downstream of the coding region.

15 Intergenic regions in the chromosome, that is to say nucleotide sequences without a coding function, can furthermore be used. Finally, prophages or defective phages contained in the chromosome can be used for this.

20 particular the genome thereof, where this is replicated together with the genome of the host and the formation of infectious particles does not take place. A defective phage is understood as meaning a prophage, in particular the genome thereof, which, as a result of various mutations, 25 has lost the ability to form so-called infectious particles. Defective phages are also called cryptic. Prophages and defective phages are often present in integrated form in the chromosome of their host. Further details exist in the prior art, for example in the textbook 30 by Edward A. Birge (Bacterial and Bacteriophage Genetics, 3<sup>rd</sup> ed., Springer-Verlag, New York, USA, 1994) or in the

To produce the coryneform bacteria according to the invention, the nucleotide sequence of the desired ORF, gene

Verlag, Jena, Germany, 1992).

textbook by S. Klaus et al. (Bakterienviren, Gustav Fischer

or allele, preferably including the expression and/or regulation signals, is isolated, at least two copies are arranged in a row, preferably in tandem arrangement, these are then transferred into the desired coryneform bacterium, preferably with the aid of vectors which do not replicate or replicate to only a limited extent in coryneform bacteria, and those bacteria in which two copies of the ORF, gene or allele are incorporated at the particular desired natural site instead of the singular copy 10 originally present are isolated, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics remaining at the 15 particular natural site (locus).

The expression and/or regulation signals mentioned, such as, for example, the ribosome binding sites, promoters, binding sites for regulatory proteins, binding sites for regulatory ribonucleic acids and attenuators lying upstream of the coding region of the ORF, gene or allele, are in general in a range of 1-800, 1-600, 1-400, 1-200, 1-100 or 1-50 nucleotides upstream of the coding region. The expression and/or regulation signals mentioned, such as, for example, the transcription terminators lying downstream of the coding region of the ORF, gene or allele, are in general in a range of 1-400, 1-200, 1-100, 1-50 or 1-25 nucleotides downstream of the coding region.

Preferably, also, no residues of sequences of the vectors used or species-foreign DNA, such as, for example,

restriction cleavage sites, remain on the flanks of the ORFs, genes or alleles amplified according to the invention. In each case a maximum of 24, preferably a maximum of 12, particularly preferably a maximum of 6 nucleotides of such DNA optionally remain on the flanks.

At least a third copy of the open reading frame (ORF), gene or allele in question is optionally inserted at a further gene site, or several further gene sites, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the further gene site.

Preferably, also, no residues of sequences of the vectors

10 used or species-foreign DNA, such as, for example,
restriction cleavage sites, remain at the further gene
site. A maximum of 24, preferably a maximum of 12,
particularly preferably a maximum of 6 nucleotides of such
DNA upstream or downstream of the ORF, gene or allele

15 incorporated optionally remain at the further gene site.

The invention accordingly also provides a process for the production of coryneform bacteria which produce one or more chemical compounds, characterized in that

- a) the nucleotide sequence of a desired ORF, gene or allele, preferably including the expression and/or regulation signals, is isolated
  - b) at least two copies of the nucleotide sequence of the ORF, gene or allele are arranged in a row, preferably in tandem arrangement
- c) the nucleotide sequence obtained according to b) is incorporated in a vector which does not replicate or replicates to only a limited extent in coryneform bacteria,
  - d) the nucleotide sequence according to b) or c) is transferred into coryneform bacteria, and

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e) coryneform bacteria which have at least two copies of the desired ORF, gene or allele at the particular

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desired natural site instead of the singular copy of the ORF, gene or allele originally present are isolated, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics remaining at the particular natural site (locus), and

at least a third copy of the open reading frame f) · 10 (ORF), gene or allele in question is optionally introduced at a further gene site, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no 15 nucleotide sequence which imparts resistance to antibiotics remaining at the further gene site.

By the measures according to the invention, the productivity of the coryneform bacteria or of the fermentative processes for the preparation of chemical compounds is improved in respect of one or more of the features chosen from the group consisting of concentration (chemical compound formed, based on the unit volume), yield (chemical compound formed, based on the source of carbon consumed) and product formation rate (chemical compound formed, based on the time) by at least 0.5 - 1.0% or at least 1.0 to 1.5% or at least 1.5 - 2.0%.

Instructions on conventional genetic engineering methods, such as, for example, isolation of chromosomal DNA, plasmid DNA, handling of restriction enzymes etc., are found in Sambrook et al. (Molecular Cloning - A Laboratory Manual (1989) Cold Spring Harbor Laboratory Press). Instructions on transformation and conjugation in coryneform bacteria are found, inter alia, in Thierbach et al. (Applied Microbiology and Biotechnology 29, 356-362 (1988)), in Schäfer et al. (Journal of Bacteriology 172, 1663-1666

(1990) and Gene 145, 69-73 (1994)) and in Schwarzer and Pühler (Bio/Technology 9, 84-87 (1991)).

Vectors which replicate to only a limited extent are understood as meaning plasmid vectors which, as a function of the conditions under which the host or carrier is cultured, replicate or do not replicate. Thus, a temperature-sensitive plasmid for coryneform bacteria which can replicate only at temperatures below 31°C has been described by Nakamura et al. (US-A-6,303,383).

- The invention also provides coryneform bacteria, in particular of the genus Corynebacterium, which produce L-lysine, characterized in that
- a) instead of the singular copy of an open reading frame (ORF), a gene or allele of lysine production

  15 naturally present at the particular desired site (locus), these have at least two copies of the said open reading frame (ORF), gene or allele, preferably in tandem arrangement, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the particular site, and in that these
- 25 b) optionally have at least a third copy of the said open reading frame (ORF), gene or allele of L-lysine production at a further gene site, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the further gene site.

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The invention also furthermore provides a process for the preparation of L-lysine, which comprises the following steps:

- a) fermentation of coryneform bacteria, in particular of the genus Corynebacterium, which
  - i) instead of the singular copy of an open reading frame (ORF), gene or allele of lysine production present at the particular desired site (locus), have at least two copies of the open reading frame (ORF), gene or allele in question, preferably in tandem arrangement, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the particular site, and in that these
  - ii) optionally have at least a third copy of the open reading frame (ORF), gene or allele of L-lysine production in question at a further gene site, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the further gene site,

under conditions which allow expression of the said open reading frames (ORFs), genes or alleles,

 concentration of the L-lysine in the fermentation broth, c) isolation of the L-lysine from the fermentation broth, optionally

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d) with constituents from the fermentation broth and/or the biomass to the extent of > (greater than) 0 to 100%.

A "copy of an open reading frame (ORF), gene or allele of lysine production" is to be understood as meaning all the, preferably endogenous, open reading frames, genes or alleles of which enhancement/over-expression can have the effect of improving lysine production. Enhancement is understood as meaning an increase in the intracellular concentration or activity of the particular gene product, protein or enzyme.

These include, inter alia, the following open reading

frames, genes or alleles: accBC, accDA, cstA, cysD, cysE,
cysH, cysK, cysN, cysQ, dapA, dapB, dapC, dapD, dapE, dapF,
ddh, dps, eno, gap, gap2, gdh, gnd, lysC, lysCFBR, lysE,
msiK, opcA, oxyR, ppc, ppcFBR, pgk, pknA, pknB, pknD, pknG,
ppsA, ptsH, ptsI, ptsM, pyc, pyc P458S, sigC, sigD, sigE,
sigH, sigM, tal, thyA, tkt, tpi, zwa1, zwf and zwf A213T.
These are summarized and explained in Table 1.

These include, in particular, the lysC<sup>FBR</sup> alleles which code for a "feed back" resistant aspartate kinase. Various lysC<sup>FBR</sup> alleles are summarized and are explained in Table 2.

The following lysC<sup>FBR</sup> alleles are preferred: lysC A279T (replacement of alanine at position 279 of the aspartate kinase protein coded, according to SEQ ID NO: 2, by threonine), lysC A279V (replacement of alanine at position 279 of the aspartate kinase protein coded, according to SEQ ID NO: 2, by valine), lysC S301F (replacement of serine at position 301 of the aspartate kinase protein coded, according to SEQ ID NO: 2, by phenylalanine), lysC T308I (replacement of threonine at position 308 of the aspartate

kinase protein coded, according to SEQ ID NO: 2, by isoleucine), lysC S301Y (replacement of serine at position 308 of the aspartate kinase protein coded, according to SEQ ID NO: 2, by tyrosine), lysC G345D (replacement of glycine at position 345 of the aspartate kinase protein coded, according to SEQ ID NO: 2, by aspartic acid), lysC R320G (replacement of arginine at position 320 of the aspartate kinase protein coded, according to SEQ ID NO: 2, by glycine), lysC T311I (replacement of threonine at position 311 of the aspartate kinase protein coded, according to SEQ ID NO: 2, by isoleucine), lysC S381F (replacement of serine at position 381 of the aspartate kinase protein coded, according to SEQ ID NO: 2, by phenylalanine).

The lysC<sup>FBR</sup> allele lysC T311I (replacement of threonine at position 311 of the aspartate kinase protein coded, according to SEQ ID NO: 2, by isoleucine), the nucleotide sequence of which is shown as SEQ ID NO:3, is particularly preferred; the amino acid sequence of the aspartate kinase protein coded is shown as SEQ ID NO:4.

- The following open reading frames, genes or nucleotide sequences, inter alia, can be used as the "further gene site" which is not essential for growth or lysine production: aecD, ccpA1, ccpA2, citA, citB, citE, fda, gluA, gluB, gluC, gluD, luxR, luxS, lysR1, lysR2, lysR3, menE, mqo, pck, pgi, poxB and zwa2, in particular the genes aecD, gluA, gluB, gluC, gluD and pck. These are summarized and explained in Table 3. Intergenic regions in the
- 30 prophages or defective phages contained in the chromosome can be used.

coding function, can furthermore be used. Finally,

chromosome, that is to say nucleotide sequences without a

 $\underline{ \mbox{Table 1}} \\ \mbox{Open reading frames, genes and alleles of lysine production}$ 

Name	Description of the coded enzyme or	Reference	Access
	protein		Number
accBC	Acyl-CoA Carboxylase	Jäger et al.	U35023
	EC 6.3.4.14	Archives of	
	(acyl-CoA carboxylase)	Microbiology	
	-	(1996) 166:76-	
		82	
		EP1108790;	AX123524
		WO0100805	AX066441
accDA	Acetyl-CoA Carboxylase	EP1055725	
	EC 6.4.1.2	EP1108790	AX121013
	(acetyl-CoA carboxylase)	WO0100805	AX066443
cstA	Carbon Starvation Protein A	EP1108790	AX120811
CSCA	(carbon starvation protein A)	WO0100804	AX066109
cysD	Sulfate Adenylyltransferase	EP1108790	AX123177
Суър	sub-unit II	EP1108/90	AXIZJIII
	EC 2.7.7.4	1	
	(sulfate adenylyltransferase small		
	chain)	mm1100700	277 2222
cysE	Serine Acetyltransferase	EP1108790	AX122902
	EC 2.3.1.30	WO0100843	AX063961
	(serine acetyltransferase)		
cysH	3'-Phosphoadenyl Sulfate Reductase		AX123178
	EC 1.8.99.4	WO0100842	AX066001
	(3'-phosphoadenosine 5'-		
	phosphosulfate reductase)		
cysK	Cysteine Synthase	EP1108790	AX122901
	EC 4.2.99.8	WO0100843	AX063963
	(cysteine synthase)		
cysN	Sulfate Adenylyltransferase sub-	EP1108790	AX123176
	unit I		AX127152
	EC 2,7.7.4	i	}
	(sulfate adenylyltransferase)		
cysQ	Transport protein CysQ	EP1108790	AX127145
	(transporter cysQ)	WO0100805	AX066423
dapA	Dihydrodipicolinate Synthase	Bonnassie et	X53993
	EC 4.2.1.52	al. Nucleic	
[	(dihydrodipicolinate synthase)	Acids Research	
1		18:6421 (1990)	1
		Pisabarro et	
Į.		al., Journal of	
}		Bacteriology	Z21502
		175:2743-	
1		2749 (1993)	1
		EP1108790	
		WO0100805	
İ		EP0435132	
		EP1067192	AX123560
		EP1067193	AX063773
dapB	Dihydrodipicolinate Reductase	EP1108790	AX127149
مريت	EC 1.3.1.26	WO0100843	AX063753
	(dihydrodipicolinate reductase)	EP1067192	AX137723
	(	EP1067192	AX137602
L		TRETOCITAD	1201010

		Pisabarro et	x67737
		al., Journal of	Z21502
		Bacteriology	
		175:2743-	
		2749 (1993)	
		JP1998215883	E16749
		JP1997322774	E14520
		JP1997070291	E12773
		JP1995075578	E08900
dapC	N-Succinyl Aminoketopimelate	EP1108790	AX127146
	Transaminase	WO0100843	AX064219
	EC 2.6.1.17	EP1136559	
	(N-succinyl diaminopimelate		
	transaminase)		
dapp	Tetrahydrodipicolinate Succinylase	EP1108790	AX127146
	EC 2.3.1.117	WO0100843	AX063757
	(tetrahydrodipicolinate	Wehrmann et al.	AJ004934
	succinylase)	Journal of	
		Bacteriology	
		180:3159-	
		3165 (1998)	
dapE	N-Succinyl Diaminopimelate	EP1108790	AX127146
	Desuccinylase	WO0100843	AX063749
	EC 3.5.1.18	Wehrmann et al.	X81379
	(N-succinyl diaminopimelate	Microbiology	
	desuccinylase)	140:3349-3356	
		(1994)	
dapF	Diaminopimelate Epimerase	EP1108790	AX127149
	EC 5.1.1.7	WO0100843	AX063719
	(diaminopimelate epimerase)	EP1085094	AX137620
ddh	Diaminopimelate Dehydrogenase	EP1108790	AX127152
	EC 1.4.1.16	WO0100843	AX063759
	(diaminopimelate dehydrogenase)	Ishino et al.,	Y00151
		Nucleic Acids	
		Research	
		15:3917-	
	1	3917 (1987)	
		JP1997322774	E14511
	•	JP1993284970	E05776
		Kim et al.,	D87976
		Journal of	
		Microbiology	
		and	
		Biotechnology	ļ
<del></del>		5:250-256(1995)	
dps	DNA Protection Protein	EP1108790	AX127153
	(protection during starvation		
	protein)	TD1100700	277.055.55
eno	Enolase	EP1108790	AX127146
	EC 4.2.1.11	WO0100844	AX064945
}	(enolase)	EP1090998	AX136862
]		Hermann et al.,	
		Electrophoresis	
		19:3217-3221	
		(1998)	
gap	Glyceraldehyde 3-Phosphate	EP1108790	AX127148
	Dehydrogenase	WO0100844	AX064941
1	EC 1.2.1.12	Eikmanns et	X59403

		<b>T</b>	
	(glyceraldehyde 3-phosphate	al., Journal of	1
	dehydrogenase)	Bacteriology	
		174:6076-	
		6086 (1992)	
gap2	Glyceraldehyde 3-Phosphate	EP1108790	AX127146
	Dehydrogenase	WO0100844	AX064939
	EC 1.2.1.12		
	(glyceraldehyde 3-phosphate	}	
	dehydrogenase 2)		
gdh	Glutamate Dehydrogenase	EP1108790	AX127150
	EC 1.4.1.4	WO0100844	AX063811
i	(glutamate dehydrogenase)	Boermann et	X59404
		al., Molecular	
		Microbiology	
		6:317-326	
		(1992).	
		Guyonvarch et	X72855
		al. NCBI	2033
gnd	6-Phosphogluconate Dehydrogenase	EP1108790	AX127147
-	EC 1.1.1.44		AX121689
	(6-phosphogluconate dehydrogenase)	WO0100844	AX065125
lysC	Aspartate Kinase	EP1108790	AX120365
-100	EC 2.7.2.4	WO0100844	AX063743
	(aspartate kinase)	Kalinowski et	X57226
	(angul out of filliant)	al., Molecular	237220
		Microbiology	
		5:1197-204	
	'	(1991)	
lysCFB	Aspartate Kinase feedback	see Table 2	
R	resistent (fbr)	see lable 2	
	EC 2.7.2.4		
	(aspartate kinase fbr)		
lysE	Lysine Exporter	EP1108790	AX123539
-3	(lysine exporter protein)	WO0100843	AX123539
	(1301110 Galporter process)	Vrljić et al.,	X96471
		Molecular	7,04/1
		Microbiology	
		22:815-826	
		(1996)	
msiK	Sugar Importer	EP1108790	AX120892
	(multiple sugar import protein)	FLITO0120	AXIZU692
орсА	Glucose 6-Phosphate Dehydrogenase	WO0104325	AX076272
,	(subunit of glucose 6-phosphate	MO0T04372	AAU10212
	dehydrogenase)		
oxyR	Transcription Regulator	ED1100700	27100100
OAYA		EP1108790	AX122198
ppcFBR	(transcriptional regulator) Phosphoenol Pyruvate Carboxylase	DD0702011	AX127149
ppe		EP0723011	
	feedback resistent	WO0100852	ĺ
	EC 4.1.1.31		-
	(phosphoenol pyruvate carboxylase		
	feedback resistant)		
ppc	Phosphoenol Pyruvate Carboxylase	EP1108790	AX127148
	EC 4.1.1.31		AX123554
	(phosphoenol pyruvate carboxylase)	O'Reagan et	M25819
		al., Gene	
		77(2):237-	
		251 (1989)	
pgk	Phosphoglycerate Kinase	EP1108790	AX121838

	EC 2.7.2.3		
ĺ	1		AX127148
	(phosphoglycerate kinase)	WO0100844	AX064943
		Eikmanns,	X59403
		Journal of	
		Bacteriology	}
		174:6076-6086	
		(1992)	
pknA	Protein Kinase A	EP1108790	AX120131
	(protein kinase A)		AX120085
pknB	Protein Kinase B	EP1108790	AX120130
	(protein kinase B)		AX120085
pknD	Protein Kinase D	EP1108790	AX127150
	(protein kinase D)		AX122469
			AX122468
pknG	Protein Kinase G	EP1108790	AX127152
	(protein kinase G)		AX123109
ppsA	Phosphoenol Pyruvate Synthase	EP1108790	AX127144
}	EC 2.7.9.2		AX120700
	(phosphoenol pyruvate synthase)		AX122469
ptsH	Phosphotransferase System Protein	EP1108790	AX122210
	н	122200750	AX127149
	EC 2.7.1.69	W00100844	AX069154
	(phosphotransferase system	1.00100044	W003134
	component H)		
ptsI	Phosphotransferase System Enzyme I	EP1108790	AX122206
	EC 2.7.3.9		AX127149
	(phosphotransferase system enzyme		AA12/149
	I)		}
ptsM	Glucose-specific	Lee et al.,	L18874
	Phosphotransferase System Enzyme	FEMS	11100/4
	II	Microbiology	
	EC 2.7.1.69	Letters 119(1-	1
	(glucose phosphotransferase-system	2):137-145	
	enzyme II)	(1994)	
рус	Pyruvate Carboxylase	W09918228	A97276
	EC 6.4.1.1	Peters-Wendisch	
	(pyruvate carboxylase)	et al.,	103340
		Microbiology	
		144:915-927	
		(1998)	
рус	Pyruvate Carboxylase	EP1108790	t
P458S	EC 6.4.1.1		
	(pyruvate carboxylase)		li
	amino acid exchange P458S		
sigC	Sigma Factor C	EP1108790	AX120368
	EC 2.7.7.6		AX120085
	(extracytoplasmic function		121220005
	alternative sigma factor C)		
sigD	RNA Polymerase Sigma Factor D	EP1108790	AX120753
	EC 2.7.7.6		AX127144
	(RNA polymerase sigma factor)	·	SAL4/144
sigE	Sigma Factor E	EP1108790	AX127146
-	EC 2.7.7.6		AX127146 AX121325
	(extracytoplasmic function	•	AVITATO
	alternative sigma factor E)		
sigH	Sigma Factor H	EP1108790	AV10714E
	EC 2.7.7.6	TT 7100130	AX127145
	(sigma factor SigH)		AX120939

sigM	Sigma Factor M	EP1108790	AX123500
_	EC 2.7.7.6		AX127153
	(sigma factor SigM)		
tal	Transaldolase	WO0104325	AX076272
	EC 2.2.1.2		
	(transaldolase)		
thyA	Thymidylate Synthase	EP1108790	AX121026
	EC 2.1.1.45		AX127145
	(thymidylate synthase)		
tkt	Transketolase	Ikeda et al.,	AB023377
	EC 2.2.1.1	NCBI	•
	(transketolase)		
tpi	Triose Phosphate Isomerase	Eikmanns,	X59403
	EC 5.3.1.1	Journal of	
	(triose phosphate isomerase)	Bacteriology	
		174:6076-6086	
<u> </u>		(1992)	3 V 1 3 3 7 0 1
zwa1	Cell Growth Factor 1	EP1111062	AX133781
	(growth factor 1)	EP1108790	AX127148
zwf	Glucose 6-Phosphate 1-	EPII08/90	AX12/148 AX121827
	Dehydrogenase	WO0104325	AX121627
		WOOT04325	AAU/02/2
	(glucose 6-phosphate 1-  dehydrogenase)		
zwf	Glucose 6-Phosphate 1-	EP1108790	
A213T	Dehydrogenase	EF1100750	}
MCT31	EC 1.1.1.49		
	(glucose 6-phosphate 1-		
	dehydrogenase)		
	amino acid exchange A213T		
L	1		

 $\frac{{\tt Table\ 2}}{{\tt lysC^{\tt FBR}}} \ {\tt alleles\ which\ code} \ {\tt for\ feed\ back\ resistant\ aspartate}$  kinases

Name of the	Amino acid	Reference	Access
allele	replacement		Number
lysCFBR-E05108		JP 1993184366-A	E05108
		(sequence 1)	
lysCFBR-E06825	lysC A279T	JP 1994062866-A	E06825
		(sequence 1)	
lysC <sup>FBR</sup> -E06826	lysC A279T	JP 1994062866-A	E06826
		(sequence 2)	
lysCFBR-E06827		JP 1994062866-A	E06827
	İ	(sequence 3)	}
lysCFBR-E08177		JP 1994261766-A	E08177
		(sequence 1)	
lysCFBR-E08178	lysC A279T	JP 1994261766-A	E08178
		(sequence 2)	
lysC <sup>FBR</sup> -E08179	lysC A279V	JP 1994261766-A	E08179
		(sequence 3)	
lysCFBR-E08180	lysC S301F	JP 1994261766-A	E08180
		(sequence 4)	
lysC <sup>FBR</sup> -E08181	lysC T308I	JP 1994261766-A	E08181
		(sequence 5)	
lysC <sup>FBR</sup> -E08182		JP 1994261766-A	E08182
lysC <sup>FBR</sup> -E12770		JP 1997070291-A	E12770
		(sequence 13)	
lysCFBR-E14514		JP 1997322774-A	E14514
		(sequence 9)	
lysC <sup>FBR</sup> -E16352		JP 1998165180-A	E16352
1777		(sequence 3)	
lysC <sup>FBR</sup> -E16745		JP 1998215883-A	E16745
TO TO TO TO TO TO TO TO TO TO TO TO TO T		(sequence 3)	
lysC <sup>FBR</sup> -E16746		JP 1998215883-A	E16746
- 500		(sequence 4)	
lysC <sup>FBR</sup> -174588		US 5688671-A	174588
- 200		(sequence 1)	
lysC <sup>FBR</sup> -I74589	lysC A279T	US 5688671-A	I74589
PDD		(sequence 2)	
lysC <sup>FBR</sup> -I74590		US 5688671-A	174590
PDS		(sequence 7)	
lysC <sup>FBR</sup> -I74591	lysC A279T	US 5688671-A	174591
7777		(sequence 8)	
lysC <sup>FBR</sup> -I74592		US 5688671-A	174592
·		(sequence 9)	

lysCFBR-I74593	13	Tra 5600655 -	T74500
TysC	lysC A279T	US 5688671-A	174593
7 PBR		(sequence 10)	
lysC <sup>FBR</sup> -174594		US 5688671-A	174594
PDD		(sequence 11)	
lysC <sup>FBR</sup> -174595	lysC A279T	US 5688671-A	174595
		(sequence 12)	
lysC <sup>FBR</sup> -I74596		US 5688671-A	174596
		(sequence 13)	
lysC <sup>FBR</sup> -I74597	lysC A279T	US 5688671-A	I74597
		(sequence 14)	
lysC <sup>FBR</sup> -X57226	lysC S301Y	EP0387527	X57226
		Kalinowski et	
		al., Molecular	
		and General	
		Genetics	
		224:317-324	
		(1990)	
lysCFBR-L16848	lysC G345D	Follettie and	L16848
_	_	Sinskey	
		NCBI Nucleotide	
		Database (1990)	
lysCFBR-L27125	lysc R320G	Jetten et al.,	L27125
-	lysC G345D	Applied	
	_	Microbiology	
		Biotechnology	
		43:76-82 (1995)	
lysCFBR	lysC T311I	W00063388	
-	_	(sequence 17)	
lysCFBR	lysC S301F	US3732144	
_			
lysCFBR	lysC S381F		<del>                                     </del>
lysCFBR		JP6261766	
1 2 2 2		(sequence 1)	
lysCFBR	lysC A279T	JP6261766	
		(sequence 2)	
lysCFBR	lysC A279V	JP6261766	<del></del>
	-100 HZ/JV	(sequence 3)	
lysC <sup>FBR</sup>	lysC S301F	JP6261766	<del> </del>
1,750	TASC SOUTE	(sequence 4)	
lysCFBR	1220 m200T	JP6261766	
TARC	lysC T308I		
	<u> </u>	(sequence 5)	<u> </u>

 $\frac{{\tt Table\ 3}}{{\tt Further\ gene\ sites}\ {\tt for\ integration\ of\ open\ reading\ frames}},$  genes and alleles of lysine production

Gene	Description of the coded	Reference	Access
name	enzyme or protein		Number
aecD	beta C-S Lyase	Rossol et al., Journal	M89931
	EC 2.6.1.1	of Bacteriology	
	(beta C-S lyase)	174(9):2968-77 (1992)	
ccpA1	Catabolite Control	WO0100844	AX065267
	Protein	EP1108790	AX127147
	(catabolite control		· ·
	protein A1)		
ccpA2	Catabolite Control	WO0100844	AX065267
	Protein	EP1108790	AX121594
	(catabolite control		
	protein A2)		
citA	Sensor Kinase CitA	EP1108790	AX120161
	(sensor kinase CitA)		
citB	Transcription Regulator	EP1108790	AX120163
	CitB		
	(transcription regulator		
	CitB)		
citE	Citrate Lyase	WO0100844	AX065421
	EC 4.1.3.6	EP1108790	AX127146
	(citrate lyase)		
fda	Fructose Bisphosphate	von der Osten et al.,	X17313
	Aldolase	Molecular Microbiology	
	EC 4.1.2.13	3(11):1625-37 (1989)	
	(fructose 1,6-		
	bisphosphate aldolase)	·	
gluA	Glutamate Transport ATP-	Kronemeyer et al.,	X81191
	binding Protein	Journal of Bacteriology	
	(glutamate transport	177(5):1152-8 (1995)	
	ATP-binding protein)		
gluB	Glutamate-binding	Kronemeyer et al.,	X81191
	Protein	Journal of Bacteriology	
	(glutamate-binding	177(5):1152-8 (1995)	
		L <del>ander, 1982</del>	

	protein)		
gluC	Glutamate Transport	Kronemeyer et al.,	X81191
	Permease	Journal of Bacteriology	V01131
	(glutamate transport	177(5):1152-8 (1995)	
1	system permease)	(1993)	
gluD	Glutamate Transport	Vronomorror of al	V01101
3242	Permease	Kronemeyer et al., Journal of Bacteriology	X81191
	(glutamate transport		
	_	177(5):1152-8 (1995)	
luxR	system permease)		
TUXK	Transcription Regulator	WO0100842	AX065953
	LuxR	EP1108790	AX123320
	(transcription regulator		
	LuxR)		
luxS	Histidine Kinase LuxS	EP1108790	AX123323
	(histidine kinase LuxS)		AX127153
lysR1	Transcription Regulator	EP1108790	AX064673
	LysR1		AX127144
; f	(transcription regulator		
	LysR1)		
lysR2	Transcription Activator	EP1108790	AX123312
	LysR2		:
	(transcription regulator		
	LysR2)		
lysR3	Transcription Regulator	WO0100842	AX065957
	LysR3	EP1108790	AX127150
	(transcription regulator		
	LysR3)		
menE	O-Succinylbenzoic Acid	WO0100843	AX064599
	CoA Ligase	EP1108790	AX064193
	EC 6.2.1.26		AX127144
	(O-succinylbenzoate CoA		
	ligase)		
mqo	Malate-Quinone	Molenaar et al., Eur.	AJ224946
	Oxidoreductase	Journal of Biochemistry	
	(malate-quinone-	1;254(2):395-403 (1998)	
	oxidoreductase)		
pck	Phosphoenol Pyruvate	WO0100844	AJ269506
	Carbonaldon		AX065053
	Carboxykinase		MAGGGGGG I

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	carboxykinase)		
pgi	Glucose 6-Phosphate	EP1087015	AX136015
	Isomerase	EP1108790	AX127146
	EC 5.3.1.9		
	(glucose-6-phosphate		
	isomerase)		
рохВ	Pyruvate Oxidase	WO0100844	AX064959
	EC 1.2.3.3	EP1096013	AX137665
	(pyruvate oxidase)		
zwa2	Cell Growth Factor 2	EP1106693	AX113822
	(growth factor 2)	EP1108790	AX127146

The invention accordingly also provides a process for the production of coryneform bacteria which produce L-lysine, characterized in that

- 5 a) the nucleotide sequence of a desired ORF, gene or allele of lysine production, optionally including the expression and/or regulation signals, is isolated
  - b) at least two copies of the nucleotide sequence of the ORF, gene or allele of lysine production are arranged in a row, preferably in tandem arrangement
    - c) the nucleotide sequence obtained according to b) is incorporated in a vector which does not replicate or replicates to only a limited extent in coryneform bacteria,
- 15 d) the nucleotide sequence according to b) or c) is transferred into coryneform bacteria, and

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e) coryneform bacteria which have at least two copies of the desired ORF, gene or allele of lysine production at the particular desired natural site instead of the singular copy of the ORF, gene or allele originally present are isolated, no nucleotide sequence which is capable of/enables episomal replication in

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microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics remaining at the particular natural site (locus), and optionally

f) at least a third copy of the open reading frame (ORF), gene or allele of lysine production in question is introduced at a further gene site, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics remaining at the further gene site.

The invention also provides coryneform bacteria, in

particular of the genus Corynebacterium, which produce Lmethionine and/or L-threonine, characterized in that

- a) instead of the singular copy of an open reading frame (ORF), a gene or allele of methionine production or threonine production naturally present at the particular desired site (locus), these have at least two copies of the said open reading frame (ORF), gene or allele, preferably in tandem arrangement, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the particular site, and in that these
- b) optionally have at least a third copy of the open reading frame (ORF), gene or allele of methionine production or threonine production mentioned at a further gene site, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is

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capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the further gene site.

The invention also furthermore provides a process for the preparation of L-methionine and/or L-threonine, which comprises the following steps:

- fermentation of coryneform bacteria, in particular of the genus Corynebacterium, which
- instead of the singular copy of an open reading i) 10 frame (ORF), gene or allele of methionine production or threonine production present at the particular desired site (locus), have at least two copies of the open reading frame (ORF), gene or allele in question, preferably in tandem 15 arrangement, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to 20 antibiotics being present at the particular site, and
  - optionally have at least a third copy of the open reading frame (ORF), gene or allele of methionine production or threonine production in question at a further gene site, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the further gene site,

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under conditions which allow expression of the

said open reading frames (ORFs), genes or alleles,

- b) concentration of the L-methionine and/or L-threonine in the fermentation broth,
- 5 c) isolation of the L-methionine and/or L-threonine from the fermentation broth, optionally
  - d) with constituents from the fermentation broth and/or the biomass to the extent of > (greater than) 0 to 100%.
- A "copy of an open reading frame (ORF), gene or allele of methionine production" is to be understood as meaning all the, preferably endogenous, open reading frames, genes or alleles of which enhancement/over-expression can have the effect of improving methionine production.
- These include, inter alia, the following open reading frames, genes or alleles: accBC, accDA, aecD, cstA, cysD, cysE, cysH, cysK, cysN, cysQ, dps, eno, fda, gap, gap2, gdh, gnd, glyA, hom, hom<sup>FBR</sup>, lysC, lysC<sup>FBR</sup>, metA, metB, metE, metH, metY, msiK, opcA, oxyR, ppc, ppc<sup>FBR</sup>, pgk, pknA, pknB,
- pknD, pknG, ppsA, ptsH, ptsI, ptsM, pyc, pyc P458S, sigC, sigD, sigE, sigH, sigM, tal, thyA, tkt, tpi, zwa1, zwf and zwf A213T. These are summarized and explained in Table 4. These include, in particular, the lysCFBR alleles which code for a "feed back" resistant aspartate kinase (see Table 2)
- and the hom<sup>FBR</sup> alleles which code for a "feed back" resistant homoserine dehydrogenase.

The at least third, optionally fourth or fifth copy of the open reading frame (ORF), gene or allele of methionine production in question can be integrated at a further site.

The following open reading frames, genes or nucleotide sequences, inter alia, can be used for this: brnE, brnF, brnQ, ccpA1, ccpA2, citA, citB, citE, ddh, gluA, gluB, gluC, gluD, luxR, luxS, lysR1, lysR2, lysR3, menE, metD,

metK, pck, pgi, poxB and zwa2. These are summarized and explained in Table 5. Intergenic regions in the chromosome, that is to say nucleotide sequences without a coding function, can furthermore be used. Finally, prophages or defective phages contained in the chromosome can be used for this.

Name	Description of the coded enzyme or	Reference	Access
	protein	North Control	Number
accBC	Acyl-CoA Carboxylase	Jäger et al.	U35023
İ	EC 6.3.4.14	Archives of	033023
	(acyl-CoA carboxylase)	Microbiology	
	( tog 1 con carbony tabe)	(1996) 166:76-82	
		EP1108790;	AX123524
		WO0100805	
accDA	Acetyl-CoA Carboxylase	EP1055725	AX066441
0000	EC 6.4.1.2	EP1035725 EP1108790	AX121013
	(acetyl-CoA carboxylase)	WO0100805	AX121013 AX066443
aecD	Cystathionine beta-Lyase	Rossol et al.,	
4000	EC 4.4.1.8	Journal of	м89931
ļ	(cystathionine beta-lyase)	Bacteriology	
	(cystathionine beta-lyase)	174:2968-2977	
		(1992)	1
cstA	Carbon Starvation Protein A		27120011
CDCA	(carbon starvation protein A)	EP1108790 WO0100804	AX120811
cysD	Sulfate Adenylyltransferase	EP1108790	AX066109 AX123177
Cyss	sub-unit II	EPI108/90	AX1231//
	EC 2.7.7.4		
	(sulfate adenylyltransferase small		
	chain)		
cysE	Serine Acetyltransferase	EP1108790	AX122902
6322	EC 2.3.1.30	WO0100843	AX122902 AX063961
	(serine acetyltransferase)	W00100943	AKUUSJUI
cysH	3'-Phosphoadenyl Sulfate Reductase	EP1108790	AX123178
-2	EC 1.8.99.4	WO0100842	AX066001
	(3'-phosphoadenosine 5'-	W00100042	AROUGUI
	phosphosulfate reductase)		
cysK	Cysteine Synthase	EP1108790	AX122901
	EC 4.2.99.8	WO0100843	AX063963
	(cysteine synthase)		1111003303
cysN	Sulfate Adenylyltransferase sub-	EP1108790	AX123176
	unit I	1211100750	AX127152
	EC 2.7.7.4		mizz, 132
	(sulfate adenylyltransferase)		1
cysQ	Transport protein CysQ	EP1108790	AX127145
	(transporter cysQ)	WO0100805	AX066423
dps	DNA Protection Protein	EP1108790	AX127153
	(protection during starvation		
	protein)		1
eno	Enolase	EP1108790	AX127146
	EC 4.2.1.11	WO0100844	AX064945
	(enolase)	EP1090998	AX136862
1	1	Hermann et al.,	m150002
		Electrophoresis	
1 .		19:3217-3221	]
		(1998)	
fda	Fructose Bisphosphate Aldolase	van der Osten et	X17313
	EC 4.1.12.13	al., Molecular	1.21,313
		/ Morecular	<u></u>

	(frugtogo highbogshaba -13-1	T	,
1	(fructose bisphosphate aldolase)	Microbiology	
		3:1625-1637	
	Clygonal dobydo 2 Phonelate	(1989)	
gap	Glyceraldehyde 3-Phosphate Dehydrogenase	EP1108790	AX127148
		WO0100844	AX064941
ļ	EC 1.2.1.12	Eikmanns et al.,	X59403
	(glyceraldehyde 3-phosphate	Journal of	
	dehydrogenase)	Bacteriology	
		174:6076-	
	Clarace debug 2 2 Di 1 1	6086 (1992)	
gap2	Glyceraldehyde 3-Phosphate Dehydrogenase	EP1108790	AX127146
'	EC 1.2.1.12	WO0100844	AX064939
			ŀ
	(glyceraldehyde 3-phosphate dehydrogenase 2)		
gdh	Glutamate Dehydrogenase	TD1100700	277407450
العطا	EC 1.4.1.4	EP1108790	AX127150
[	(glutamate dehydrogenase)	WO0100844	AX063811
	(grutamate demydrogenase)	Boermann et al.,	X59404
		Molecular	
		Microbiology	
		6:317-326 (1992)	*****
ļ		Guyonvarch et al,	X72855
glyA	Glycine/Serine	NCBI	77107146
91yr	Hydroxymethyltransferase	EP1108790	AX127146
	EC 2.1.2.1		AX121194
}	(glycine/serine		
	hydroxymethyltransferase)		
gnd	6-Phosphogluconate Dehydrogenase	EP1108790	AV107147
giid	EC 1.1.1.44	EP1108/90	AX127147 AX121689
	(6-phosphogluconate dehydrogenase)	WO0100844	AX121689 AX065125
hom	Homoserine Dehydrogenase	Peoples et al.,	Y00546
	EC 1.1.1.3	Molecular	100546
	(homoserine dehydrogenase)	Microbiology	1
	, and a second and an organization,	2:63-72 (1988)	1
homFBR	Homoserine Dehydrogenase feedback	Reinscheid et	<del> </del>
	resistant (fbr)	al., Journal of	
	EC 1.1.1.3	Bacteriology	
	(homoserine dehydrogenase fbr)	173:3228-30	
		(1991)	
lysC	Aspartate Kinase	EP1108790	AX120365
-	EC 2.7.2.4	WO0100844	AX063743
	(aspartate kinase)	Kalinowski et	X57226
		al., Molecular	
		Microbiology	
		5:1197-204 (1991)	
lysCFB	Aspartate Kinase feedback	see Table 2	<u> </u>
R	resistent (fbr)		
	EC 2.7.2.4		
	(aspartate kinase fbr)		
metA	Homoserine Acetyltransferase	Park et al.,	AF052652
	EC 2.3.1.31	Molecular Cells	
	(homoserine acetyltransferase)	8:286-94 (1998)	1
metB	Cystathionine \( \gamma\)-Lyase	Hwang et al.,	AF126953
	EC 4.4.1.1	Molecular Cells	111120933
1	(cystathionine gamma-synthase)	9:300-308 (1999)	
metE	Homocysteine Methyltransferase	EP1108790	27127146
	EC 2.1.1.14	   PLTTA012A	AX127146
	TO 4.1.4.12	<u> </u>	AX121345

- Т	(homocysteine methyltransferase)		
metH	Homocysteine Methyltransferase	EP1108790	AX127148
	(Vitamin B12-dependent)	1111100730	AX121747
	EC 2.1.1.14		
	(homocysteine methyltransferase)		1
metY	Acetylhomoserine Sulfhydrolase	EP1108790	AX120810
	(acetylhomoserine sulfhydrolase)	22200.30	AX127145
msiK	Sugar Importer	EP1108790	AX120892
	(multiple sugar import protein)	212200,30	
opcA	Glucose 6-Phosphate Dehydrogenase	WO0104325	AX076272
	(subunit of glucose 6-phosphate		
	dehydrogenase)		
oxyR	Transcription Regulator	EP1108790	AX122198
-	(transcriptional regulator)		AX127149
ppcFBR	Phosphoenol Pyruvate Carboxylase	EP0723011	
	feedback resistent	WO0100852	
	EC 4.1.1.31		
	(phosphoenol pyruvate carboxylase	•	
	feedback resistant)		
ppc	Phosphoenol Pyruvate Carboxylase	EP1108790	AX127148
	EC 4.1.1.31		AX123554
	(phosphoenol pyruvate carboxylase)	O'Reagan et al.,	M25819
		Gene 77(2):237-	
		251 (1989)	
pgk	Phosphoglycerate Kinase	EP1108790	AX121838
	EC 2.7.2.3		AX127148
	(phosphoglycerate kinase)	WO0100844	AX064943
	•	Eikmanns, Journal	X59403
		of Bacteriology	
		174:6076-6086	
		(1992)	2000000
pknA	Protein Kinase A	EP1108790	AX120131
pknB	(protein kinase A) Protein Kinase B	EP1108790	AX120085 AX120130
pkns	(protein kinase B)	EP1108/90	AX120130
pknD	Protein Kinase D	EP1108790	AX127150
DVID	(protein kinase D)	EP1108/90	AX127150
	(protein kinase b)		AX122468
pknG	Protein Kinase G	EP1108790	AX127152
paid	(protein kinase G)	EFIIO0790	AX123109
ppsA	Phosphoenol Pyruvate Synthase	EP1108790	AX127144
Ppon	EC 2.7.9.2	121100750	AX120700
ŀ	(phosphoenol pyruvate synthase)		AX122469
ptsH	Phosphotransferase System Protein	EP1108790	AX122210
	H		AX127149
	1	WO0100844	AX069154
	EC 2.7.1.69	INCOTOGGAA	THEOLOGIC
	EC 2.7.1.69 (phosphotransferase system	100100044	I I I I I I I I I I I I I I I I I I I
L		M001000##	TAROUS 25 2
ptsI	(phosphotransferase system		AX122206
ptsI	(phosphotransferase system component H)		
ptsI	(phosphotransferase system component H) Phosphotransferase System Enzyme I		AX122206
ptsI	(phosphotransferase system component H)  Phosphotransferase System Enzyme I EC 2.7.3.9		AX122206
ptsI ptsM	(phosphotransferase system component H)  Phosphotransferase System Enzyme I EC 2.7.3.9 (phosphotransferase system enzyme		AX122206
	(phosphotransferase system component H)  Phosphotransferase System Enzyme I EC 2.7.3.9 (phosphotransferase system enzyme I)	EP1108790	AX122206 AX127149
	(phosphotransferase system component H)  Phosphotransferase System Enzyme I EC 2.7.3.9 (phosphotransferase system enzyme I)  Glucose-specific Phosphotransferase System Enzyme II	Lee et al., FEMS Microbiology Letters 119(1-	AX122206 AX127149
	(phosphotransferase system component H)  Phosphotransferase System Enzyme I EC 2.7.3.9 (phosphotransferase system enzyme I)  Glucose-specific Phosphotransferase System Enzyme II EC 2.7.1.69	EP1108790  Lee et al., FEMS Microbiology	AX122206 AX127149
	(phosphotransferase system component H)  Phosphotransferase System Enzyme I EC 2.7.3.9 (phosphotransferase system enzyme I)  Glucose-specific Phosphotransferase System Enzyme II	Lee et al., FEMS Microbiology Letters 119(1-	AX122206 AX127149

		Tricont cons	20000
рус	Pyruvate Carboxylase	WO9918228	A97276
	EC 6.4.1.1	Peters-Wendisch	Y09548
	(pyruvate carboxylase)	et al.,	
		Microbiology	
	•	144:915-927	
		(1998)	ļ ·
рус	Pyruvate Carboxylase	EP1108790	
P458S	EC 6.4.1.1		
	(pyruvate carboxylase)		
	amino acid exchange P458S	<u> </u>	
sigC	Sigma Factor C	EP1108790	AX120368
	EC 2.7.7.6		AX120085
	(extracytoplasmic function		
	alternative sigma factor C)		
sigD	RNA Polymerase Sigma Factor D	EP1108790	AX120753
	EC 2.7.7.6		AX127144
	(RNA polymerase sigma factor)		<u> </u>
sigE	Sigma Factor E	EP1108790	AX127146
	EC 2.7.7.6		AX121325
	(extracytoplasmic function		
<u> </u>	alternative sigma factor E)		
sigH	Sigma Factor H	EP1108790	AX127145
	EC 2.7.7.6		AX120939
	(sigma factor SigH)		
sigM	Sigma Factor M	EP1108790	AX123500
	EC 2.7.7.6		AX127153
	(sigma factor SigM)		
tal	Transaldolase	WO0104325	AX076272
	EC 2.2.1.2		
	(transaldolase)		
thyA	Thymidylate Synthase	EP1108790	AX121026
	EC 2.1.1.45		AX127145
	(thymidylate synthase)		
tkt	Transketolase	Ikeda et al.,	AB023377
	EC 2.2.1.1	NCBI	
	(transketolase)	,	
tpi	Triose Phosphate Isomerase	Eikmanns, Journal	X59403
	EC 5.3.1.1	of Bacteriology	
	(triose phosphate isomerase)	174:6076-6086	1
		(1992)	20000
zwa1	Cell Growth Factor 1	EP1111062	AX133781
<u></u>	(growth factor 1)		277 054 45
zwf	Glucose 6-Phosphate 1-	EP1108790	AX127148
	Dehydrogenase		AX121827
	EC 1.1.1.49	WO0104325	AX076272
	(glucose 6-phosphate 1-		
	dehydrogenase)		-
zwf	Glucose 6-Phosphate 1-	EP1108790	
A213T			
	EC 1.1.1.49		
	(glucose 6-phosphate 1-	1	1
	dehydrogenase)		1
	amino acid exchange A213T		J

 $\frac{{\tt Table}\ 5}{{\tt Further}\ {\tt gene}\ {\tt sites}\ {\tt for}\ {\tt integration}\ {\tt of}\ {\tt open}\ {\tt reading}\ {\tt frames},}$  genes and alleles of methionine production

Description of the	Peferonce	Access
	kererence	
coded enzyme or procern		Number
Transporter of	EP1096010	AX137709
		AX137714
acids		AA13//14
(branched-chain amino		
acid transporter)	·	
Transporter of	EP1096010	AX137709
		AX137714
	1	
	}	
		M89931
		AX066841
	169(4):303-12 (1998)	AX127150
	F	
	EP1108790	
		AX065267
	EP1108790	AX127147
		ļ <u>.</u>
		AX065267
<del></del>	EP1108790	AX121594
	P744 00500	
	EP1108790	AX120161
	ED1100700	3 2 2 0 0 1 6 2
	EP1108/90	AX120163
		]
	WO0100844	AX065421
		AX127146
Diaminopimelate	Ishino et al., Nucleic	S07384
Dehydrogenase	Acids Research 15: 3917	AX127152
EC 1.4.1.16	(1987)	
(diaminopimelate	EP1108790	,
dehydrogenase)		
Glutamate Transport	Kronemeyer et al.,	X81191
ATP-binding Protein	Journal of Bacteriology	
(glutamate transport	177(5):1152-8 (1995)	
ATP-binding protein)		
Glutamate-binding	Kronemeyer et al.,	X81191
Protein	Journal of Bacteriology	
(glutamate-binding		]
(Aracamace princing		
protein)		
· ·	Kronemeyer et al., Journal of Bacteriology	X81191
	(branched-chain amino acid transporter) Transporter of branched-chain amino acids (branched-chain amino acids (branched-chain amino acid transporter) Carrier protein of branched-chain amino acids (branched-chain amino acids (branched-chain amino acid transport system carrier protein) Catabolite Control Protein (catabolite control protein A1) Catabolite Control Protein (catabolite control protein (catabolite control protein A2) Sensor Kinase CitA (sensor kinase CitA) Transcription Regulator CitB (transcription regulator CitB) Citrate Lyase EC 4.1.3.6 (citrate lyase) Diaminopimelate Dehydrogenase EC 1.4.1.16 (diaminopimelate dehydrogenase) Glutamate Transport ATP-binding Protein (glutamate transport ATP-binding protein) Glutamate-binding	Transporter of branched-chain amino acids (branched-chain amino acid transporter)  Carrier protein of branched-chain amino acid transport system carrier protein)  Catabolite Control Protein Al)  Catabolite

(glutamate transport system permease) Glutamate Transport Permease (glutamate transport	177(5):1152-8 (1995)  Kronemeyer et al.,	X81191
Glutamate Transport Permease	Kronemeyer et al.,	X81191
Permease		
(glutamate transport	Journal of Bacteriology	
	177(5):1152-8 (1995)	1
system permease)	(2,722)	
Transcription Regulator	W00100842	AX065953
LuxR	EP1108790	AX123320
(transcription		12225555
regulator LuxR)		
Histidine Kinase LuxS	EP1108790	AX123323
(histidine kinase LuxS)		AX127153
Transcription Regulator	EP1108790	AX064673
LysR1		AX127144
(transcription	_	
regulator LysR1)		
Transcription Activator	EP1108790	AX123312
LysR2		
(transcription		
regulator LysR2)		
Transcription Regulator	WO0100842	AX065957
LysR3	EP1108790	AX127150
(transcription		
regulator LysR3)		
O-Succinylbenzoic Acid	W00100843	AX064599
	EP1108790	AX064193
		AX127144
	EP1108790	AX123327
		AX127153
regulator MetD)		
	· · ·	AX063959
	EP1108790	AX127148
	W001 000 4 4	
	WOOTOO844	AJ269506
		AX065053
	ED1007015	27126015
		AX136015
	ELT108/30	AX127146
	W00100844	24064050
_		AX064959
	ELT020012	AX137665
	ED1106602	237112222
		AX113822 AX127146
	Histidine Kinase LuxS (histidine kinase LuxS) Transcription Regulator LysR1 (transcription regulator LysR1) Transcription Activator LysR2 (transcription regulator LysR2) Transcription Regulator LysR3 (transcription	Histidine Kinase LuxS (histidine kinase LuxS)  Transcription Regulator LysR1 (transcription regulator LysR1)  Transcription Activator LysR2 (transcription regulator LysR2)  Transcription Regulator LysR3 (transcription regulator LysR3)  O-Succinylbenzoic Acid CoA Ligase EC 6.2.1.26 (O-succinylbenzoate CoA ligase)  Transcription Regulator MetD (transcription regulator MetD) Methionine Adenosyl Transferase EC 2.5.1.6 (S-adenosylmethionine synthetase) Phosphoenol Pyruvate Carboxykinase (phosphoenol pyruvate carboxykinase) Glucose 6-Phosphate Isomerase EC 5.3.1.9 (glucose-6-phosphate isomerase) Pyruvate Oxidase EP1108790  EP1108790  W00100843 EP1108790  W00100844  EP1087015 EP1108790  EP108790  EP1108790  EP108790  EP1108790  EP1108

A "copy of an open reading frame (ORF), gene or allele of threonine production" is to be understood as meaning all the, preferably endogenous, open reading frames, genes or alleles of which enhancement/over-expression can have the effect of improving threonine production.

These include, inter alia, the following open reading frames, genes or alleles: accBC, accDA, cstA, cysD, cysE, cysH, cysI, cysN, cysQ, dps, eno, fda, gap, gap2, gdh, gnd, hom, hom<sup>FBR</sup>, lysC, lysC<sup>FBR</sup>, msiK, opcA, oxyR, ppc, ppc<sup>FBR</sup>, pgk, pknA, pknB, pknD, pknG, ppsA, ptsH, ptsI, ptsM, pyc, pyc P458S, sigC, sigD, sigE, sigH, sigM, tal, thyA, tkt, tpi, thrB, thrC, thrE, zwa1, zwf and zwf A213T. These are summarized and explained in Table 6. These include, in particular, the lysC<sup>FBR</sup> alleles which code for a "feed back" resistant aspartate kinase (See Table 2) and the hom<sup>FBR</sup> alleles which code for a "feed back" resistant homoserine dehydrogenase.

The at least third, optionally fourth or fifth copy of the open reading frame (ORF), gene or allele of threonine 20 production in question can be integrated at a site. The following open reading frames, genes or nucleotide sequences, inter alia, can be used for this: ccpA1, ccpA2, citA, citB, citE, ddh, gluA, gluB, gluC, gluD, glyA, ilvA, ilvBN, ilvC, ilvD, luxR, luxS, lysR1, lysR2, lysR3, mdh, 25 menE, metA, metD, pck, poxB, sigB and zwa2. These are summarized and explained in Table 7. Intergenic regions in the chromosome, that is to say nucleotide sequences without a coding function, can furthermore be used. Finally, prophages or defective phages contained in the chromosome 30 can be used for this.

 $\frac{{\tt Table}\ 6}{{\tt Open}\ {\tt reading}\ {\tt frames},\ {\tt genes}\ {\tt and}\ {\tt alleles}\ {\tt of}\ {\tt threonine}}$   ${\tt production}$ 

Name	Description of the coded enzyme or	Reference	Access
	protein	, and the same	Number
	protein		Number
accBC	Acyl-CoA Carboxylase	Jäger et al.	U35023
	EC 6.3.4.14	Archives of	
	(acyl-CoA carboxylase)	Microbiology	
	(4032 0011 011200113 2420)	(1996) 166:76-	
		82	AX123524
		EP1108790;	AX066441
		W00100805	
accDA	Acetyl-CoA Carboxylase	EP1055725	
	EC 6.4.1.2	EP1108790	AX121013
	(acetyl-CoA carboxylase)	WO0100805	AX066443
cstA	Carbon Starvation Protein A	EP1108790	AX120811
	(carbon starvation protein A)	W00100804	AX066109
cysD	Sulfate Adenylyltransferase	EP1108790	AX123177
-	sub-unit II		
	EC 2.7.7.4	<u> </u>	
	(sulfate adenylyltransferase small		
	chain)		
cysE	Serine Acetyltransferase	EP110.8790	AX122902
	EC 2.3.1.30	WO0100843	AX063961
	(serine acetyltransferase)		
cysH	3'-Phosphoadenyl Sulfate Reductase	EP1108790	AX123178
	EC 1.8.99.4	WO0100842	AX066001
·	(3'-phosphoadenosine 5'-phosphosulfate		:
	reductase)		
cysK	Cysteine Synthase	EP1108790	AX122901
	EC 4.2.99.8	WO0100843	AX063963
	(cysteine synthase)		
cysN	Sulfate Adenylyltransferase sub-unit I	EP1108790	AX123176
ļ	EC 2.7.7.4		AX127152
<u> </u>	(sulfate adenylyltransferase)		
cysQ	Transport protein CysQ	EP1108790	AX127145
	(transporter cysQ)	WO0100805	AX066423
dps	DNA Protection Protein	EP1108790	AX127153
	(protection during starvation protein)		
eno	Enolase	EP1108790	AX127146
	EC 4.2.1.11	WO0100844	AX064945
	(enolase)	EP1090998	AX136862
		Hermann et al.,	]
		Electrophoresis	
		19:3217-3221	
£3-	Fructose Bisphosphate Aldolase	(1998)	V17212
fda		van der Osten	X17313
	EC 4.1.2.13	et al., Molecular	
	(fructose bisphosphate aldolase)	Microbiology	
		3:1625-1637	
		(1989)	
Can	Glyceraldehyde 3-Phosphate Dehydrogenase		AX127148
gap	1 gracerardenade 2-knosbuare nenadrodeugse	I ELTTO 130	TUVT 7 1 1 4 9

	I		
	EC 1.2.1.12	WO0100844	AX064941
	(glyceraldehyde 3-phosphate	Eikmanns et	X59403
}	dehydrogenase)	al., Journal of	
		Bacteriology	
1		174:6076-	
		6086(1992)	
gap2	Glyceraldehyde 3-Phosphate Dehydrogenase	EP1108790	AX127146
i	EC 1.2.1.12	WO0100844	AX064939
ļ	(glyceraldehyde 3-phosphate		
	dehydrogenase 2)		
gdh	Glutamate Dehydrogenase	EP1108790	AX127150
_	EC 1.4.1.4	WO0100844	AX063811
	(glutamate dehydrogenase)	Boermann et	X59404
	, ign = 1 and 1 an	al., Molecular	V23404
		Microbiology 6:317-326	
		(1992)	¥700EE
		, ,	X72855
		Guyonvarch et al, NCBI	1
gnd	6-Phosphogluconate Dehydrogenase		37700740
9.14	EC 1.1.1.44	EP1108790	AX127147
	(6-phosphogluconate dehydrogenase)	TYO 0.1 0.0 0.4 4	AX121689
hom	Homoserine Dehydrogenase	WO0100844 Peoples et al.,	AX065125
110111	EC 1.1.1.3	Molecular	Y00546
	(homoserine dehydrogenase)	Microbiology	}
	(nomoberine denydrogenase)		
homFBR	Homoserine Dehydrogenase feedback	2:63-72 (1988) Reinscheid et	
110111	resistant (fbr)		
	EC 1.1.1.3	al., Journal of	
	(homoserine dehydrogenase fbr)	Bacteriology	
	(nomoserine denydrogenase rbr)	173:3228-30	
lysC	Aspartate Kinase	(1991)	
1320	EC 2.7.2.4	EP1108790	AX120365
		WO0100844	AX063743
	(aspartate kinase)	Kalinowski et	X57226
		al., Molecular	
		Microbiology	
		5:1197-204	
lysCFBR	7	(1991)	
TARC	Aspartate Kinase feedback resistent	see Table 2	
	(fbr)		
	EC 2.7.2.4		
mo-! 77	(aspartate kinase fbr)		
msiK	Sugar Importer	EP1108790	AX120892
07.73	(multiple sugar import protein)		
opcA	Glucose 6-Phosphate Dehydrogenase	WO0104325	AX076272
	(subunit of glucose 6-phosphate		
	dehydrogenase)		
oxyR	Transcription Regulator	EP1108790	AX122198
gag	(transcriptional regulator)		AX127149
ppcFBR	Phosphoenol Pyruvate Carboxylase	EP0723011	
	feedback resistent	WO0100852	}
	EC 4.1.1.31		i
	(phosphoenol pyruvate carboxylase		l
	feedback resistant)		
ppc	Phosphoenol Pyruvate Carboxylase	EP1108790	AX127148
	EC 4.1.1.31		AX123554
	(phosphoenol pyruvate carboxylase)	O'Reagan et	M25819
		al., Gene	

$\overline{}$		177/03 037	
		77(2):237-	
	The make a large to the state of the state o	251 (1989)	217101020
pgk	Phosphoglycerate Kinase	EP1108790	AX121838
	EC 2.7.2.3		AX127148
	(phosphoglycerate kinase)	WO0100844	AX064943
		Eikmanns,	X59403
		Journal of	
		Bacteriology	
		174:6076-6086 (1992)	
pknA	Protein Kinase A	EP1108790	AX120131
PKIIA	(protein kinase A)	ELTT08/30	AX120131 AX120085
pknB	Protein Kinase B	EP1108790	
PKUB	(protein kinase B)	EP1108/90	AX120130
pknD	Protein Kinase D	EP1108790	AX120085
PKID	(protein kinase D)	EP1108/90	AX127150 AX122469
	(procein kinase D)		AX122469 AX122468
pknG	Protein Kinase G	EP1108790	AX127152
pkiid	(protein kinase G)	EP1100/30	AX127152 AX123109
ppsA	Phosphoenol Pyruvate Synthase	EP1108790	AX123109 AX127144
PPSA	EC 2.7.9.2	E1100/30	AX127144 AX120700
	(phosphoenol pyruvate synthase)		AX120700
ptsH	Phosphotransferase System Protein H	EP1108790	AX122210
pcsn	EC 2.7.1.69	EF1100/30	AX127149
	(phosphotransferase system component H)	W00100844	AX069154
ptsI	Phosphotransferase System Enzyme I	EP1108790	AX122206
PCSI	EC 2.7.3.9	BETTOOTO	AX127149
	(phosphotransferase system enzyme I)		11112/143
ptsM	Glucose-specific Phosphotransferase	Lee et al.,	L18874
	System Enzyme II	FEMS	
Ì	EC 2.7.1.69	Microbiology	1
Ì	(glucose phosphotransferase-system	Letters 119(1-	
	enzyme II)	2):137-145	
		(1994)	
рус	Pyruvate Carboxylase	W09918228	A97276
	EC 6.4.1.1	Peters-Wendisch	Y09548
	(pyruvate carboxylase)	et al.,	
		Microbiology	İ
		144:915-927	İ
		(1998)	
рус	Pyruvate Carboxylase	EP1108790	ł
P458S	EC 6.4.1.1		1
	(pyruvate carboxylase)		Ì
	amino acid exchange P458S		
sigC	Sigma Factor C	EP1108790	AX120368
	EC 2.7.7.6		AX120085
ļ	(extracytoplasmic function alternative		
<u></u>	sigma factor C)		
sigD	RNA Polymerase Sigma Factor D	EP1108790	AX120753
	EC 2.7.7.6		AX127144
	(RNA polymerase sigma factor)	ED1100700	27100146
sigE	Sigma Factor E	EP1108790	AX127146
	EC 2.7.7.6		AX121325
	(extracytoplasmic function alternative sigma factor E)		
	Sigma Factor H	EP1108790	AX127145
sigH	EC 2.7.7.6	PLT100/20	AX12/145 AX120939
	(sigma factor SigH)		HA120333
1	(SIGNE FACEOF SIGN)	1	

sigM	Sigma Factor M	EP1108790	AX123500
•	EC 2.7.7.6		AX127153
	(sigma factor SigM)		
tal	Transaldolase	WO0104325	AX076272
	EC 2.2.1.2		
	(transaldolase)		
thrB	Homoserine Kinase	Peoples et al.,	Y00546
	EC 2.7.1.39	Molecular	
	(homoserine kinase)	Microbiology	
		2:63-72 (1988)	J
thrC	Threonine Synthase	Han et al.,	X56037
	EC 4.2.99.2	Molecular	
	(threonine synthase)	Microbiology	
		4:1693-1702	
		(1990)	
thrE	Threonine Exporter	EP1085091	AX137526
	(threonine export carrier)		
thyA	Thymidylate Synthase	EP1108790	AX121026
	EC 2.1.1.45		AX127145
	(thymidylate synthase)		
tkt	Transketolase	Ikeda et al.,	AB023377
	EC 2.2.1.1	NCBI.	
	(transketolase)		X59403
tpi	Triose Phosphate Isomerase	Eikmanns, Journal of	X59403
	EC 5.3.1.1	-	
	(triose phosphate isomerase)	Bacteriology 174:6076-6086	
		(1992)	
		EP1111062	AX133781
zwal	Cell Growth Factor 1	EPITITOOZ	AALJJIOL
zwf	(growth factor 1) Glucose 6-Phosphate 1-Dehydrogenase	EP1108790	<del> </del>
ZWI	EC 1.1.1.49	121100750	
	(glucose 6-phosphate 1-dehydrogenase)	W00104325	
1	(Gracose a-bitosphace r denigarogenase)		
zwf	Glucose 6-Phosphate 1-Dehydrogenase	EP1108790	AX127148
A213T	EC 1.1.1.49		AX121827
Naiji	(glucose 6-phosphate 1-dehydrogenase)		AX076272
	amino acid exchange A213T		

 $\frac{{\tt Table}\ 7}{{\tt Further}\ {\tt gene}\ {\tt sites}\ {\tt for}\ {\tt integration}\ {\tt of}\ {\tt open}\ {\tt reading}\ {\tt frames},}$  genes and alleles of threonine production

Gene	Description of the coded	Reference	1.
name	enzyme or protein	Reference	Access
	chayme of procein		Number
ccpA1	Catabolite Control	1,1001,000,11	ļ.,
CCDAI	Protein	W00100844	AX065267
		EP1108790	AX127147
	(catabolite control protein A1)		
ccpA2	Catabolite Control	WO0100844	AX065267
	Protein	EP1108790	AX121594
	(catabolite control		
	protein A2)		
citA	Sensor Kinase CitA	EP1108790	AX120161
	(sensor kinase CitA)		
citB	Transcription Regulator	EP1108790	AX120163
	CitB		
İ	(transcription regulator		
	CitB)		
citE	Citrate Lyase	WO0100844	AX065421
	EC 4.1.3.6	EP1108790	AX127146
	(citrate lyase)		12127210
ddh	Diaminopimelate	Ishino et al., Nucleic	S07384
	Dehydrogenase	Acids Research 15: 3917	AX127152
	EC 1.4.1.16	(1987)	12127132
	(diaminopimelate	EP1108790	
	dehydrogenase)		1
gluA	Glutamate Transport ATP-	Kronemeyer et al.,	X81191
	binding Protein	Journal of Bacteriology	101151
	(glutamate transport ATP-	177(5):1152-8 (1995)	
	binding protein)	(1,1220)	
gluB	Glutamate-binding Protein	Kronemeyer et al.,	X81191
	(glutamate-binding	Journal of Bacteriology	MOTIFI
	protein)	177(5):1152-8 (1995)	
gluC	Glutamate Transport	Kronemeyer et al.,	X81191
_	Permease	Journal of Bacteriology	201191
1	(glutamate transport	177(5):1152-8 (1995)	ĺ
	system permease)	1	
gluD	Glutamate Transport	Kronemeyer et al.,	X81191
<b>-</b>	Permease	Journal of Bacteriology	VOTTAT
	(glutamate transport	177(5):1152-8 (1995)	ì
	system permease)	177(3):1132-8 (1995)	
glyA	Glycine	W00100843	
3-1-1	Hydroxymethyltransferase	WOOT00843	AX063861
	EC 2.1.2.1		AF327063
	(glycine		ĺ
ilvA	hydroxymethyltransferase)	362 3 3	
TTAM	Threonine Dehydratase	Möckel et al., Journal	A47044
	EC 4.2.1.16	of Bacteriology 174	L01508
	(threonine dehydratase)	(24), 8065-8072 (1992)	AX127150
		EP1108790	
ilvBN	Acetolactate Synthase	Keilhauer et al.,	A48648
	EC 4.1.3.18	Journal of Bacteriology	L09232

	(acetolactate synthase)	175(17):5595-603 (1993) EP1108790	AX127147
ilvC	Reductoisomerase	Keilhauer et al.,	C48648
1110	EC 1.1.1.86	Journal of Bacteriology	AX127147
	(ketol-acid	175 (17):5595-603 (1993)	
	reductoisomerase)	EP1108790	
ilvD	Dihydroxy-acid	EP1006189	AX136925
TIAD	Dehydratase	Briodicy	121230323
	EC 4.2.1.9		ŀ
	(dihydroxy-acid		
	dehydratase)		
luxR	Transcription Regulator	W00100842	AX065953
TUXK	LuxR	EP1108790	AX123320
	(transcription regulator	EF1100750	HILLESSEO
	· -		
J	LuxR) Histidine Kinase LuxS	EP1108790	AX123323
luxS	(histidine kinase LuxS)	EP1108/90	AX123323
3 54	(nistidine kinase Luxs)	EP1108790	AX064673
lysR1	Transcription Regulator	EP1108/90	AX127144
	LysR1		AX12/144
	(transcription regulator		İ
	LysR1)	771100700	AX123312
lysR2	Transcription Activator	EP1108790	AATZSSTZ
	LysR2		
	(transcription regulator		
	LysR2)	1	DYOCE OF T
lysR3	Transcription Regulator	W00100842	AX065957 AX127150
	LysR3	EP1108790	AXIZ/ISO
	(transcription regulator		
	LysR3)	1	AX064895
mdh	Malate Dehydrogenase	WO0100844	AXU64895
	EC 1.1.1.37		
	(malate dehydrogenase)	1:00100043	AX064599
menE	0-Succinylbenzoic Acid	WO0100843	AX064193
	CoA Ligase	EPI108/90	AX127144
	EC 6.2.1.26		WY17/144
	(O-succinylbenzoate CoA	1	
	ligase)	Park et al., Molecular	AX063895
metA	Homoserine O-		AX127145
	Acetyltransferase	Cells 30;8(3):286-94 (1998)	WV17/142
	EC 2.3.1.31		
	(homoserine O-	WO0100843 EP1108790	
	acetyltransferase)		AX123327
metD	Transcription Regulator	EP1108790	AX123327
	MetD		AXIZ/153
	(transcription regulator		
	MetD)		77260506
pck	Phosphoenol Pyruvate	WO0100844	AJ269506
	Carboxykinase		AX065053
	(phosphoenol pyruvate		1
	carboxykinase)		7.770.540.50
рохВ	Pyruvate Oxidase	WO0100844	AX064959
	EC 1.2.3.3	EP1096013	AX137665
	(pyruvate oxidase)		<del> </del>
sigB	RNA Polymerase	EP1108790	AX127149
1	Transcription Factor		1
	(RNA polymerase		1
	transcription factor)		
zwa2	Cell Growth Factor 2	EP1106693	AX113822

(growth factor 2) EP1108790 AX127146

The invention accordingly also provides a process for the production of coryneform bacteria which produce L-methionine and/or L-threonine, characterized in that

- 5 a) the nucleotide sequence of a desired ORF, gene or allele of methionine production or threonine production, optionally including the expression and/or regulation signals, is isolated
- b) at least two copies of the nucleotide sequence of the
  ORF, gene or allele of methionine production or
  threonine production are arranged in a row, preferably
  in tandem arrangement
  - c) the nucleotide sequence obtained according to b) is incorporated in a vector which does not replicate or replicates to only a limited extent in coryneform bacteria,
  - d) the nucleotide sequence according to b) or c) is transferred into coryneform bacteria, and
- e) coryneform bacteria which have at least two copies of
  the desired ORF, gene or allele of methionine or
  threonine production at the particular desired natural
  site instead of the singular copy of the ORF, gene or
  allele originally present are isolated, no nucleotide
  sequence which is capable of/enables episomal
- replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics remaining at the particular natural site (locus), and optionally
- 30 f) at least a third copy of the open reading frame (ORF), gene or allele of methionine production or threonine

production in question is introduced at a further gene site, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics remaining at the further gene site.

The invention also provides coryneform bacteria, in particular of the genus Corynebacterium, which produce L-valine, characterized in that

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- a) instead of the singular copy of an open reading frame (ORF), a gene or allele of valine production naturally present at the particular desired site (locus), these have at least two copies of the said open reading frame (ORF), gene or allele, preferably in tandem arrangement, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the particular site, and in that these
- b) optionally have at least a third copy of the open reading frame (ORF), gene or allele of valine production mentioned at a further gene site, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the further gene site.
- 30 The invention also furthermore provides a process for the preparation of L-valine, which comprises the following steps:

- a) fermentation of coryneform bacteria, in particular of the genus Corynebacterium, which
- i) instead of the singular copy of an open reading frame (ORF), gene or allele of valine production present at the particular desired site (locus), have at least two copies of the open reading frame (ORF), gene or allele in question, preferably in tandem arrangement, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the particular site, and

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- ii) optionally have at least a third copy of the open
  reading frame (ORF), gene or allele of valine
  production in question at a further gene site, no
  nucleotide sequence which is capable of/enables
  episomal replication in microorganisms, no
  nucleotide sequence which is capable of/enables
  transposition and no nucleotide sequence which
  imparts resistance to antibiotics being present at
  the further gene site,
- under conditions which allow expression of the said open reading frames (ORFs), genes or alleles,
  - b) concentration of the L-valine in the fermentation broth,
  - c) isolation of the L-valine from the fermentation broth, optionally
- 30 d) with constituents from the fermentation broth and/or the biomass to the extent of > (greater than) 0 to 100%.

A "copy of an open reading frame (ORF), gene or allele of valine production" is to be understood as meaning all the, preferably endogenous, open reading frames, genes or alleles of which enhancement/over-expression can have the

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PCT/EP02/08465

WO 03/014330

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These include, inter alia, the following open reading frames, genes or alleles: brnE, brnF, brnEF, cstA, cysD, dps, eno, fda, gap, gap2, gdh, ilvB, ilvN, ilvBN, ilvC, ilvD, ilvE msiK, pgk, ptsH, ptsI, ptsM, sigC, sigD, sigE, sigH, sigM, tpi and zwal. These are summarized and explained in Table 8. These include in particular the acetolactate synthase ilvBN alleles which code for a valine-resistant.

effect of improving valine production.

The at least third, optionally fourth or fifth copy of the
open reading frame (ORF), gene or allele of valine
production in question can be integrated at a further site.
The following open reading frames, genes or nucleotide
sequences, inter alia, can be used for this: aecD, ccpA1,
ccpA2, citA, citB, citE, ddh, gluA, gluB, gluC, gluD, glyA,
ilvA, luxR, lysR1, lysR2, lysR3, panB, panC, poxB and zwa2.
These are summarized and explained in Table 9. Intergenic
regions in the chromosome, that is to say nucleotide
sequences without a coding function, can furthermore be
used. Finally, prophages or defective phages contained in
the chromosome can be used for this.

Table 8

Open reading frames, genes and alleles of valine production

Name	Description of the coded enzyme or	Reference	Access
1102.10	protein	1.0202020	Number
]	process.		-,
brnEF	Export of branched-chain amino	EP1096010	
	acids		
i	(branched chain amino acid export)	Kennerknecht et	AF454053
]	(branched chain amino acid exporc)	al., NCBI	W. 42 4022
cstA	Carbon Starvation Protein A	EP1108790	AX120811
CSCA	(carbon starvation protein A)	WO0100804	AX066109
2	DNA Protection Protein	EP1108790	AX127153
dps		EP1108/90	AVIZ 1122
	(protection during starvation		
	protein) Enolase	EP1108790	AX127146
eno	EC 4.2.1.11	WO0100844	AX064945
			AX136862
	(enolase)	EP1090998	AX130002
		Hermann et al.,	
		Electrophoresis	
		(1998)	X17313
fda	Fructose Bisphosphate Aldolase	van der Osten et al., Molecular	VT 12T2
	EC 4.1.2.13		
	(fructose bisphosphate aldolase)	Microbiology	
		3:1625-1637	
		(1989)	27127140
gap	Glyceraldehyde 3-Phosphate	EP1108790	AX127148 AX064941
	Dehydrogenase	WO0100844	X59403
	EC 1.2.1.12	Eikmanns et al., Journal of	A39403
	(glyceraldehyde 3-phosphate	-	Ì
	dehydrogenase)	Bacteriology	
ļ			,
	Glasses I debade 2 Phoenhote	6086(1992) EP1108790	AX127146
gap2	Glyceraldehyde 3-Phosphate	WO0100844	AX064939
İ	Dehydrogenase EC 1.2.1.12	WOO100844	ANOUGOO
ł			1
	(glyceraldehyde 3-phosphate		
	dehydrogenase 2)   Glutamate Dehydrogenase	EP1108790	AX127150
gdh	EC 1.4.1.4	WO0100844	AX063811
	4 = <del>-</del>	Boermann et al.,	X59404
	(glutamate dehydrogenase)	Molecular	A33404
		Microbiology	
		6:317-326	l .
			1
		(1992); Guyonvarch et	x72855
		-	A/4055
11	Destalaghaba Gashirasa	al., NCBI Keilhauer et	L09232
ilvBN	Acetolactate Synthase	al., Journal of	DO3737
	EC 4.1.3.18	Bacteriology	
	(acetolactate synthase)		
		175 (17):5595-603	
		(1993) EP1108790	AV127147
<del></del>	T	<del></del>	AX127147
ilvC	Isomeroreductase	Keilhauer et	C48648
L	EC 1.1.1.86	al., Journal of	AX127147

			<del></del>
	(acetohydroxy acid	Bacteriology	
<b></b>	isomeroreductase)	175(17):5595-603	
		(1993)	
		EP1108790	
ilvD	Dihydroxy-acid Dehydratase	EP1006189	AX136925
	EC 4.2.1.9		
	(dihydroxy acid dehydratase)		
ilvE	Transaminase B	EP1108790	AX127150
	EC 2.6.1.42		AX122498
	(transaminase B)		
msiK	Sugar Importer	EP1108790	AX120892
1	(multiple sugar import protein)		
pgk	Phosphoglycerate Kinase	EP1108790	AX121838
pgk	EC 2.7.2.3		AX127148
İ	(phosphoglycerate kinase)	WO0100844	AX064943
1	(phobphogal octave manage)	Eikmanns,	X59403
	}	Journal of	
		Bacteriology	
•		174:6076-6086	
		(1992)	
ptsH	Phosphotransferase System Protein H	EP1108790	AX122210
pesn	EC 2.7.1.69		AX127149
	(phosphotransferase system	WO0100844	AX069154
ì	component H)	""	
ptsI	Phosphotransferase System Enzyme I	EP1108790	AX122206
ptsi	EC 2.7.3.9		AX127149
	(phosphotransferase system enzyme	1	
1	(phosphociansierase system enzyme		
ptsM	Glucose-specific Phosphotransferase	Lee et al., FEMS	L18874
PCSM	System Enzyme II	Microbiology	
1	EC 2.7.1.69	Letters 119(1-	ł
	(glucose phosphotransferase-system	2):137-145	
	enzyme II)	(1994)	
sigC	Sigma Factor C	EP1108790	AX120368
sige	EC 2.7.7.6		AX120085
ì	(extracytoplasmic function		
	alternative sigma factor C)		
sigD	RNA Polymerase Sigma Factor D	EP1108790	AX120753
Sign	EC 2.7.7.6		AX127144
i	(RNA polymerase sigma factor)		
sigE	Sigma Factor E	EP1108790	AX127146
bran	EC 2.7.7.6		AX121325
	(extracytoplasmic function	1	
	alternative sigma factor E)		
sigH	Sigma Factor H	EP1108790	AX127145
ard	EC 2.7.7.6		AX120939
1	(sigma factor SigH)		
sigM	Sigma Factor M	EP1108790	AX123500
PIGM	EC 2.7.7.6		AX127153
	(sigma factor SigM)		
	Triose Phosphate Isomerase	Eikmanns,	X59403
tpi	EC 5.3.1.1	Journal of	
	(triose phosphate isomerase)	Bacteriology	
	(CITOSE PHOSPHACE ISOMETASE)	174:6076-6086	
		(1992)	
	Cell Growth Factor 1	EP1111062	AX133781
zwa1			
	(growth factor 1)		

Table 9

Further gene sites for integration of open reading frames,
genes and alleles of valine production

Gene	Description of the coded	Reference	Access
name	enzyme or protein	Reference	Number
manie	enzyme or procern	•	Number
aecD	beta C-S Lyase	Rossol et al., Journal	M89931
accb	EC 2.6.1.1	of Bacteriology	1105552
1	(beta C-S lyase)	174(9):2968-77 (1992)	
ccpA1	Catabolite Control	WO0100844	AX065267
CCDAI	Protein	EP1108790	AX127147
	(catabolite control	BELLOOTO	ARIZITAI
	protein A1)		İ
ccpA2	Catabolite Control	WO0100844	AX065267
CCDAZ	Protein	EP1108790	AX121594
<b> </b>	(catabolite control	B11100750	MILLISSA
1	protein A2)		i
citA	Sensor Kinase CitA	EP1108790	AX120161
CILA		EP1108/30	AAIZUIUI
-147	(sensor kinase CitA) Transcription Regulator	EP1108790	AX120163
citB	CitB	EP1100/90	AAIZUIUS
	(transcription regulator		1
	CitB)		
citE	Citrate Lyase	WO0100844	AX065421
CILE	EC 4.1.3.6	EP1108790	AX127146
	(citrate lyase)	EFIIO0750	ALLETITO
ddh	Diaminopimelate	Ishino et al., Nucleic	507384
dan	Dehydrogenase	Acids Research 15: 3917	AX127152
	EC 1.4.1.16	(1987)	12.12.1
ł	(diaminopimelate	EP1108790	
	dehydrogenase)	511100750	
gluA	Glutamate Transport ATP-	Kronemeyer et al.,	X81191
grun	binding Protein	Journal of Bacteriology	
i	(glutamate transport ATP-	177(5):1152-8 (1995)	1
	binding protein)		
gluB	Glutamate-binding Protein	Kronemeyer et al.,	X81191
gran	(glutamate-binding	Journal of Bacteriology	
	protein)	177(5):1152-8 (1995)	
gluC	Glutamate Transport	Kronemeyer et al.,	X81191
9240	Permease	Journal of Bacteriology	
I	(glutamate transport	177(5):1152-8 (1995)	1
	system permease)		
gluD	Glutamate Transport	Kronemeyer et al.,	X81191
3	Permease	Journal of Bacteriology	
	(glutamate transport	177(5):1152-8 (1995)	}
	system permease)	, , , , , , , , , , , , , , , , , , , ,	
glyA	Glycine	WO0100843	AX063861
3-1	Hydroxymethyltransferase		AF327063
	EC 2.1.2.1		
	(glycine		]
	hydroxymethyltransferase)		]
ilvA	Threonine Dehydratase	Möckel et al., Journal	A47044
	EC 4.2.1.16	of Bacteriology 174	L01508
	(threonine dehydratase)	(24), 8065-8072 (1992)	AX127150

		EP1108790	<del></del>
luxR	Transcription Regulator LuxR (transcription regulator LuxR)	W00100842 EP1108790	AX065953 AX123320
lysR1	Transcription Regulator LysR1 (transcription regulator LysR1)	EP1108790	AX064673 AX127144
lysR2	Transcription Activator LysR2 (transcription regulator LysR2)	EP1108790	AX123312
lysR3	Transcription Regulator LysR3 (transcription regulator LysR3)	W00100842 EP1108790	AX065957 AX127150
panB	Ketopantoate Hydroxymethyltransferase EC 2. 1. 2. 11 (ketopantoate hydroxymethyltransferase)	US6177264	X96580
panC	Pantothenate Synthetase EC 6.3.2.1 (pantothenate synthetase)	US6177264	X96580
рохВ	Pyruvate Oxidase EC 1.2.3.3 (pyruvate oxidase)	WO0100844 EP1096013	AX064959 AX137665
zwa2	Cell Growth Factor 2 (growth factor 2)	EP1106693 EP1108790	AX113822 AX127146

The invention accordingly also provides a process for the production of coryneform bacteria which produce L-valine, characterized in that

- 5 a) the nucleotide sequence of a desired ORF, gene or allele of valine production, optionally including the expression and/or regulation signals, is isolated
  - b) at least two copies of the nucleotide sequence of the ORF, gene or allele of valine production are arranged in a row, preferably in tandem arrangement

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c) the nucleotide sequence obtained according to b) is incorporated in a vector which does not replicate or replicates to only a limited extent in coryneform bacteria,

- d) the nucleotide sequence according to b) or c) is transferred into coryneform bacteria, and
- e) coryneform bacteria which have at least two copies of the desired open ORF, gene or allele of valine

  5 production at the particular desired natural site instead of the singular copy of the ORF, gene or allele originally present are isolated, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics remaining at the particular natural site (locus), and optionally
- f) at least a third copy of the open reading frame (ORF), gene or allele of valine production in question is introduced at a further gene site, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics remaining at the further gene site.

During work on the present invention, it was possible to incorporate two copies, arranged in tandem, of an lysC<sup>FBR</sup> allele at the lysC gene site of Corynebacterium glutamicum such that no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics remain at the lysC gene site. Such a strain is, for example, the strain

30 DSM13992lysC<sup>FBR</sup>::lysC<sup>FBR</sup>.

The plasmid pK18mobsacB2xlysCSma2/1, with the aid of which two copies of an lysC<sup>FBR</sup> allele can be incorporated into the lysC gene site of Corynebacterium glutamicum, is shown in Figure 1.

During work on the present invention, it was furthermore possible to incorporate two copies, arranged in tandem, of the lysE gene at the lysE gene site of Corynebacterium glutamicum such that no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics remained at the lysE gene site. Such a strain is, for example, the strain

10 ATCC21513\_171ysE::lysE.

A plasmid with the aid of which two copies of an lysE gene can be incorporated into the lysE gene site of Corynebacterium glutamicum is shown in Figure 2. It carries the name pK18mobsacB2xlysESma1/1.

- During work on the present invention, finally, it was possible to incorporate two copies, arranged in tandem, of the zwal gene at the zwal gene site of Corynebacterium glutamicum such that no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics remained at the zwal gene site. Such a strain is, for example, the strain ATCC21513\_17zwal::zwal.
- 25 A plasmid with the aid of which two copies of a zwal gene can be incorporated into the zwal gene site of Corynebacterium glutamicum is shown in Figure 3. It carries the name pK18mobsacBzwalzwal.

The coryneform bacteria produced according to the invention can be cultured continuously or discontinuously in the batch process (batch culture) or in the fed batch (feed process) or repeated fed batch process (repetitive feed process) for the purpose of production of chemical compounds. A summary of known culture methods is described

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in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).

The culture medium to be used must meet the requirements of the particular strains in a suitable manner. Descriptions of culture media for various microorganisms are contained in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981).

Sugars and carbohydrates, such as e.g. glucose, sucrose, lactose, fructose, maltose, molasses, starch and cellulose, oils and fats, such as e.g. soya oil, sunflower oil, groundnut oil and coconut fat, fatty acids, such as e.g. palmitic acid, stearic acid and linoleic acid, alcohols, such as e.g. glycerol and ethanol, and organic acids, such as e.g. acetic acid or lactic acid, can be used as the source of carbon. These substances can be used individually or as a mixture.

Organic nitrogen-containing compounds, such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soya bean flour and urea, or inorganic compounds, such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate, can be used as the source of nitrogen. The sources of nitrogen can be used individually or as a mixture.

Phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts can be used as the source of phosphorus. The culture medium must furthermore comprise salts of metals, such as e.g. magnesium sulfate or iron sulfate, which are necessary for growth. Finally, essential growth substances, such as amino acids and vitamins, can be

employed in addition to the above-mentioned substances. Suitable precursors can moreover be added to the culture medium. The starting substances mentioned can be added to the culture in the form of a single batch, or can be fed in during the culture in a suitable manner.

Basic compounds, such as sodium hydroxide, potassium hydroxide, ammonia or aqueous ammonia, or acid compounds, such as phosphoric acid or sulfuric acid, can be employed in a suitable manner to control the pH of the culture.

Antifoams, such as e.g. fatty acid polyglycol esters, can be employed to control the development of foam. Suitable substances having a selective action, such as e.g. antibiotics, can be added to the medium to maintain the stability of plasmids. To maintain aerobic conditions,

oxygen or oxygen-containing gas mixtures, such as e.g. air, are introduced into the culture. The temperature of the culture is usually 20°C to 45°C, and preferably 25°C to 40°C. Culturing is continued until a maximum of the desired chemical compound has formed. This target is usually reached within 10 hours to 160 hours.

It has been found that the coryneform bacteria according to the invention, in particular the coryneform bacteria which produce L-lysine, have an unexpectedly high stability. They were stable for at least 10-20, 20-30, 30-40, 40-50, preferably at least 50-60, 60-70, 70-80 and 80-90

The following microorganisms have been deposited:

generations or cell division cycles.

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The Corynebacterium glutamicum strain

DSM13992lysC<sup>FBR</sup>::lysC<sup>FBR</sup> was deposited in the form of a pure

culture on 5th June 2002 under number DSM15036 at the

Deutsche Sammlung für Mikroorganismen und Zellkulturen

(DSMZ, Braunschweig, Germany) in accordance with the

Budapest Treaty.

The plasmid pK18mobsacB2xlysCSma2/1 was deposited in the form of a pure culture of the strain E. coli DH50mcr/pK18mobsacB2xlysCSma2/1 (= DH5alphamcr/pK18mobsacB2xlysCSma2/1) on 20th April 2001 under number DSM14244 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) in accordance with the Budapest Treaty.

The Corynebacterium glutamicum strain
ATCC21513\_171ysE::lysE was deposited in the form of a pure
culture on 5th June 2002 under number DSM15037 at the
Deutsche Sammlung für Mikroorganismen und Zellkulturen
(DSMZ, Braunschweig, Germany) in accordance with the
Budapest Treaty.

The Corynebacterium glutamicum strain

15 ATCC21513\_17zwa1::zwa1 was deposited in the form of a pure culture on 5th June 2002 under number DSM15038 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) in accordance with the Budapest Treaty.

## 20 Example 1

Generation of a tandem duplication of the lysC<sup>FBR</sup> allele lysC T311I in the chromosome of Corynebacterium glutamicum

- 1.1. Construction of the tandem vector pK18mobsacB2xlysCSma2/1
- 25 From the Corynebacterium glutamicum strain DSM13994, chromosomal DNA is isolated by the conventional methods (Eikmanns et al., Microbiology 140: 1817 1828 (1994)).

The strain DSM13994 was produced by multiple, non-directed mutagenesis, selection and mutant selection from C.

30 glutamicum ATCC13032. The strain is resistant to the lysine analogue S-(2-aminoethyl)-L-cysteine and has a feed back-resistant aspartate kinase which is insensitive to

inhibition by a mixture of lysine and threonine (in each case 25 mM). The nucleotide sequence of the lysCFBR allele is shown as SEQ ID NO:3. It is also called lysC T311I in the following. The amino acid sequence of the aspartate kinase protein coded is shown as SEQ ID NO:4. A pure culture of this strain was deposited on 16th January 2001 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) in accordance with the Budapest Treaty.

10 With the aid of the polymerase chain reaction, a DNA section which carries the lysC gene or allele is amplified. On the basis of the sequence of the lysC gene known for C. glutamicum (Kalinowski et al., Molecular Microbiology, 5 (5), 1197 - 1204(1991); Accession Number X57226), the following primer oligonucleotides were chosen for the PCR:

lysC1beg (SEQ ID No: 15):

5 TA (G GAT CC) T CCG GTG TCT GAC CAC GGT G 3

lysC2end: (SEQ ID NO: 16):

5 AC (G GAT CC) G CTG GGA AAT TGC GCT CTT CC 3

The primers shown are synthesized by MWG Biotech and the PCR reaction is carried out by the standard PCR method of Innis et al. (PCR Protocols. A Guide to Methods and Applications, 1990, Academic Press). The primers allow amplification of a DNA section of approx. 1.7 kb in length, which carries the lysC gene or allele. The primers moreover contain the sequence for a cleavage site of the restriction endonuclease BamHI, which is marked by parentheses in the nucleotide sequence shown above.

The amplified DNA fragment of approx. 1.7 kb in length
which carries the lysC<sup>FBR</sup> allele lysC T311I of the strain
DSM13994 is identified by electrophoresis in a 0.8% agarose
gel, isolated from the gel and purified by conventional
methods (QIAquick Gel Extraction Kit, Qiagen, Hilden).

Ligation of the fragment is then carried out by means of the Topo TA Cloning Kit (Invitrogen, Leek, The Netherlands, Cat. Number K4600-01) in the vector pCRII-TOPO. The ligation batch is transformed in the E. coli strain TOP10 (Invitrogen, Leek, The Netherlands). Selection of plasmid-carrying cells is made by plating out the transformation batch on kanamycin (50 mg/l)-containing LB agar with X-Gal (5-bromo-4-chloro-3-indolyl  $\beta$ -D-galactopyranoside, 64 mg/l).

The plasmid obtained is checked by means of restriction cleavage, after isolation of the DNA, and identified in agarose gel. The resulting plasmid is called pCRIITOPOlysC.

The nucleotide sequence of the amplified DNA fragment or PCR product is determined by the dideoxy chain termination method of Sanger et al. (Proceedings of the National Academy of Sciences USA, 74:5463-5467 (1977)) using the "ABI Prism 377" sequencing apparatus of PE Applied Biosystems (Weiterstadt, Germany). The sequence of the coding region of the PCR product is shown in SEQ ID No:3. The amino acid sequence of the associated aspartate kinase protein is shown in SEQ ID NO:4.

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The base thymine is found at position 932 of the nucleotide sequence of the coding region of the lysC<sup>FBR</sup> allele of strain DSM13994 (SEQ ID NO:3). The base cytosine is found at the corresponding position of the wild-type gene (SEQ ID NO:1).

The amino acid isoleucine is found at position 311 of the amino acid sequence of the aspartate kinase protein of strain DSM13994 (SEQ ID No:4). The amino acid threonine is found at the corresponding position of the wild-type protein (SEQ ID No:2).

The lysC allele, which contains the base thymine at position 932 of the coding region and accordingly codes for

an aspartate kinase protein which contains the amino acid isoleucine at position 311 of the amino acid sequence, is called the lysC<sup>FBR</sup> allele lysC T311I in the following.

The plasmid pCRIITOPOlysC, which carries the lysC<sup>FBR</sup> allele lysC T311I, was deposited in the form of a pure culture of the strain E. coli TOP 10/pCRIITOPOlysC under number DSM14242 on 20th April 2001 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) in accordance with the Budapest Treaty.

10

Plasmid DNA was isolated from the strain DSM14242, which carries the plasmid pCRIITOPOlysC, and cleaved with the restriction enzyme BamHI (Amersham-Pharmacia, Freiburg, Germany), after separation in an agarose gel (0.8%) the lysCFBR-containing DNA fragment approx. 1.7 kb long is 15 isolated from the agarose gel with the aid of the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), and the overhanging ends are completed with Klenow polymerase (Boehringer Mannheim) and employed for ligation with the mobilizable cloning vector pK18mobsacB described by Schäfer 20 et al., Gene, 14, 69-73 (1994). This is cleaved beforehand with the restriction enzyme SmaI and dephosphorylated with alkaline phosphatase (Alkaline Phosphatase, Boehringer Mannheim), mixed with the lysCFBR-containing fragment of approx. 1.7 kb and the mixture is treated with T4 DNA 25 Ligase (Amersham-Pharmacia, Freiburg, Germany).

The E. coli strain DH5α (Grant et al.; Proceedings of the National Academy of Sciences USA, 87 (1990) 4645-4649) is then transformed with the ligation batch (Hanahan, In. DNA Cloning. A Practical Approach. Vol. 1, ILR-Press, Cold Spring Harbor, New York, 1989). Selection of plasmid-carrying cells is made by plating out the transformation batch on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2<sup>nd</sup> Ed., Cold Spring Harbor, New York, 1989), which was supplemented with 25 mg/l kanamycin.

Plasmid DNA is isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by restriction cleavage with the enzyme HindIII and subsequent agarose gel electrophoresis. The plasmid is called pK18mobsacB1xlysCSma2.

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In a second step, the plasmid pCRII-TOPOlysC is in turn cleaved with the restriction enzyme BamHI (Amersham-Pharmacia, Freiburg, Germany), after separation in an agarose gel (0.8%) the lysCFBR-containing fragment of 10 approx. 1.7 kb was isolated from the agarose gel with the aid of the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and employed for ligation with the vector pK18mobsacB1xlysCSma2 described in this Example. This is cleaved beforehand with the restriction enzyme BamHI and 15 dephosphorylated with alkaline phosphatase (Alkaline Phosphatase, Boehringer Mannheim), mixed with the lysCFBRcontaining fragment of approx. 1.7 kb and the mixture is treated with T4 DNA Ligase (Amersham-Pharmacia, Freiburg, Germany).

The E. coli strain DH5α (Grant et al.; Proceedings of the National Academy of Sciences USA, 87 (1990) 4645-4649) is then transformed with the ligation batch (Hanahan, In. DNA Cloning. A Practical Approach. Vol. 1, ILR-Press, Cold Spring Harbor, New York, 1989). Selection of plasmid-carrying cells is made by plating out the transformation batch on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2<sup>nd</sup> Ed., Cold Spring Harbor, New York, 1989), which was supplemented with 25 mg/l kanamycin.

Plasmid DNA is isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by restriction cleavage with the enzyme HindIII and subsequent agarose gel electrophoresis. The plasmid is called pK18mobsacB2xlysCSma2/1. A map of the plasmid is shown in Figure 1.

The plasmid pK18mobsacB2xlysCSma2/1 was deposited in the form of a pure culture of the strain E. coli DH50mcr/pK18mobsacB2xlysCSma2/1 (= DH5alphamcr/pK18mobsacB2xlysCSma2/1) on 20th April 2001 under number DSM14244 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) in accordance with the Budapest Treaty.

- 1.2. Generation of a tandem duplication of the lysC<sup>FBR</sup> allele lysC T311I in C. glutamicum strain DSM13992
- 10 The vector pK18mobsacB2xlysCSma2/1 mentioned in Example 1.1 is transferred by a modified protocol of Schäfer et al. (1990 Journal of Microbiology 172: 1663-1666) into the C. glutamicum strain DSM13992.

The Corynebacterium glutamicum strain DSM13992 was produced

by multiple, non-directed mutagenesis, selection and mutant
selection from C. glutamicum ATCC13032. The strain is
resistant to the antibiotic streptomycin and phenotypically
resistant to the lysine analogue S-(2-aminoethyl)-Lcysteine. However, the strain has a wild-type aspartate

kinase (see SEQ ID NO:1 and 2), which is sensitive to
inhibition by a mixture of lysine and threonine (in each
case 25 mM). A pure culture of this strain was deposited on
16th January 2001 at the Deutsche Sammlung für
Mikroorganismen und Zellkulturen (DSMZ, Braunschweig,

Germany) in accordance with the Budapest Treaty.

The vector pK18mobsacB2xlysCSma2/1 cannot replicate independently in DSM13992 and is retained in the cell only if it has integrated into the chromosome.

Selection of clones with integrated pK18mobsacB2xlysCSma2/1
is carried out by plating out the conjugation batch on LB
agar (Sambrook et al., Molecular Cloning: A Laboratory
Manual. 2<sup>nd</sup> Ed., Cold Spring Harbor, New York, 1989), which
was supplemented with 15 mg/l kanamycin and 50 mg/l

nalidixic acid. Clones which have grown on are plated out on LB agar plates with 25 mg/l kanamycin and incubated for 16 hours at 33°C. To achieve excision of the plasmid with only one copy of the lysC gene, the clones are cultured on LB agar with 10% sucrose, after incubation for 16 hours in LB liquid medium. The plasmid pK18mobsacB contains a copy of the sacB gene, which converts sucrose into levan sucrase, which is toxic to C. glutamicum.

Only those clones in which the pK18mobsacB2xlysCSma2/1

integrated has been excised again therefore grow on LB agar with sucrose. Approximately 40 to 50 colonies are tested for the phenotype "growth in the presence of sucrose" and "non-growth in the presence of kanamycin". During the excision, either two copies of the lysC gene or only one can be excised together with the plasmid.

To demonstrate that two copies of lysC have remained in the chromosome, approximately 20 colonies which show the phenotype "growth in the presence of sucrose" and "nongrowth in the presence of kanamycin" are investigated with the aid of the polymerase chain reaction by the standard PCR method of Innis et al. (PCR Protocols. A Guide to Methods and Applications, 1990, Academic Press). A DNA fragment which carries the lysC gene and surrounding regions is amplified here from the chromosomal DNA of the colonies. The following primer oligonucleotides are chosen for the PCR.

lysck1 (SEQ ID NO: 5):

5 TCG GTG TCA TCA GAG CAT TG 3 

lysck2 (SEQ ID NO: 6):

5 TCG GTT GCC TGA GTA ATG TC 3

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The primers allow amplification of a DNA fragment approx.

1.9 kb in size in control clones with the original lysC locus. In clones with a second copy of the lysC gene in the

chromosome at the lysC locus, DNA fragments with a size of approx. 3.6 kb are amplified.

The amplified DNA fragments are identified by means of electrophoresis in a 0.8% agarose gel. On the basis of the amplified fragment length, a distinction was made between clones with one chromosomal lysC gene copy and clones with two chromosomal lysC gene copies.

10 clones with two complete copies of the lysC gene on the chromosome are investigated with the aid of the LightCycler of Roche Diagnostics (Mannheim, Germany) in order to demonstrate whether the two copies are lysC<sup>FBR</sup> alleles with the mutation lysC T311I or whether the original wild-type lysC is present alongside an lysC<sup>FBR</sup> allele lysC T311I. The LightCycler is a combined apparatus of Thermocycler and flourimeter.

A DNA section approx. 500 bp in length which contains the mutation site is amplified in the first phase by means of a PCR (Innis et al., PCR Protocols. A Guide to Methods and Applications, 1990, Academic Press) using the following primer oligonucleotides.

```
LC-lysC1-fbr (SEQ ID No: 7):
5` aaccgttctgggtatttccg 3`
LC-lysC2-fbr (SEQ ID No: 8):
5` tccatgaactctgcggtaac 3`
```

- In the second phase, with two additional oligonucleotides of different lengths and marked with different fluorescent dyestuffs (Lightcycler(LC)-Red640 and fluorescein), which hybridize in the region of the mutation site, the presence of the mutation is detected with the aid of the
- 30 "Fluorescence Resonance Energy Transfer" method (FRET) using a melting curve analysis (Lay et al., Clinical Chemistry, 43:2262-2267 (1997)).

lysC311-C (SEQ ID No: 9):

5 LC-Red640 - gcaggtgaagatgatgtcggt - (P) 3

lysC311-A (SEQ ID No: 10):

- 5` tcaagatctccatcgcgcggcggccgtcggaacga fluorescein 3`
- 5 The primers shown are synthesized for the PCR by MWG Biotech and oligonucleotides shown for the hybridization are synthesized by TIB MOLBIOL (Berlin, Germany).

A clone which contains the base thymine at position 932 of the coding regions of the two lysC copies and thus has two 10 lysC<sup>FBR</sup> alleles lysC T311I was identified in this manner.

The strain was called C. glutamicum DSM139921ysCFBR::lysCFBR.

The strain was deposited as C. glutamicum DSM13992lysCFBR::lysCFBR on 5th June 2002 under number DSM15036 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) in accordance with the Budapest Treaty.

## Example 2

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Generation of a tandem duplication of the lysE gene in the chromosome of Corynebacterium glutamicum

20 2.1. Construction of the tandem vector pK18mobsacB2xlysESma1/1

Plasmid DNA was isolated from the Escherichia coli strain DSM12871 (EP-A-1067193), which carries the plasmid pEC7lysE.

The plasmid contains the lysE gene which codes for lysine export. A pure culture of this strain was deposited on 10th June 1999 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) in accordance with the Budapest Treaty.

The plasmid pEC71lysE is cleaved with the restriction enzyme BamHI (Amersham-Pharmacia, Freiburg, Germany), after separation in an agarose gel (0.8%) the lysE fragment of approx. 1.1 kb is isolated from the agarose gel with the aid of the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), and the overhanging ends are completed with Klenow polymerase (Boehringer Mannheim) and employed for ligation with the mobilizable cloning vector pK18mobsacB described by Schäfer et al., Gene, 14, 69-73 (1994). This is cleaved beforehand with the restriction enzyme SmaI and dephosphorylated with alkaline phosphatase (Alkaline Phosphatase, Boehringer Mannheim), mixed with the lysE fragment of approx. 1.1 kb and the mixture is treated with T4 DNA Ligase (Amersham-Pharmacia, Freiburg, Germany).

The E. coli strain DH5α (Grant et al.; Proceedings of the National Academy of Sciences USA, 87 (1990) 4645-4649) is then transformed with the ligation batch (Hanahan, In. DNA Cloning. A Practical Approach. Vol. 1, ILR-Press, Cold Spring Harbor, New York, 1989). Selection of plasmid-carrying cells is made by plating out the transformation batch on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2<sup>nd</sup> Ed., Cold Spring Harbor, New York, 1989), which was supplemented with 25 mg/l kanamycin.

Plasmid DNA is isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by restriction cleavage with the enzymes BamHI and EcoRI and subsequent agarose gel electrophoresis. The plasmid is called pK18mobsacBlxlysESma1.

In a second step, the plasmid pEC7lysE is in turn cleaved

with the restriction enzyme BamHI (Amersham-Pharmacia,
Freiburg, Germany), after separation in an agarose gel

(0.8%) the lysE fragment of approx. 1.1 kb was isolated
from the agarose gel with the aid of the QIAquick Gel
Extraction Kit (Qiagen, Hilden, Germany) and employed for

ligation with the vector pK18mobsacB1xlysESma1 described in

this Example. This is cleaved beforehand with the restriction enzyme BamHI and dephosphorylated with alkaline phosphatase (Alkaline Phosphatase, Boehringer Mannheim), mixed with the lysE fragment of approx. 1.1 kb and the mixture is treated with T4 DNA Ligase (Amersham-Pharmacia, Freiburg, Germany).

The E. coli strain DH5α (Grant et al.; Proceedings of the National Academy of Sciences USA, 87 (1990) 4645-4649) is then transformed with the ligation batch (Hanahan, In. DNA Cloning. A Practical Approach. Vol. 1, ILR-Press, Cold Spring Harbor, New York, 1989). Selection of plasmid-carrying cells is made by plating out the transformation batch on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2<sup>nd</sup> Ed., Cold Spring Harbor, New York, 1989), which was supplemented with 25 mg/l kanamycin.

Plasmid DNA is isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by restriction cleavage with the enzymes EcoRI and SalI or ScaI and subsequent agarose gel electrophoresis. The plasmid is called pK18mobsacB2xlysESma1/1. A map of the plasmid is shown in Figure 2.

2.2. Generation of a tandem duplication of the lysE gene in C. glutamicum strain ATCC21513\_17

The vector pK18mobsacB2xlysESma1/1 mentioned in Example 2.1 is transferred by a modified protocol of Schäfer et al. (1990 Journal of Microbiology 172: 1663-1666) into the C. glutamicum strain ATCC21513\_17.

The Corynebacterium glutamicum strain ATCC21513\_17 was produced by multiple, non-directed mutagenesis, selection and mutant selection from C. glutamicum ATCC21513. The strain is resistant to the lysine analogue S-(2-aminoethyl)-L-cysteine and both leucine- and homoserine-prototrophic.

The vector cannot replicate independently in ATCC21513\_17 and is retained in the cell only if it has integrated into the chromosome.

Selection of clones with integrated pK18mobsacB2xlysESma1/1
is carried out by plating out the conjugation batch on LB
agar (Sambrook et al., Molecular Cloning: A Laboratory
Manual. 2<sup>nd</sup> Ed., Cold Spring Harbor, New York, 1989), which
was supplemented with 15 mg/l kanamycin and 50 mg/l
nalidixic acid. Clones which have grown on are plated out
on LB agar plates with 25 mg/l kanamycin and incubated for
16 hours at 33°C. To achieve excision of the plasmid with
only one copy of the lysE gene, the clones are cultured on
LB agar with 10% sucrose, after incubation for 16 hours in
LB liquid medium. The plasmid pK18mobsacB contains a copy
of the sacB gene, which converts sucrose into levan
sucrase, which is toxic to C. glutamicum.

Only those clones in which the pK18mobsacB2xlysESma1/1 integrated has been excised again therefore grow on LB agar with sucrose. Approximately 40 to 50 colonies are tested for the phenotype "growth in the presence of sucrose" and "non-growth in the presence of kanamycin". During the excision, either two copies of the lysE gene or only one can be excised together with the plasmid.

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To demonstrate that two copies of lysE have remained in the

25 chromosome, approximately 20 colonies which show the
phenotype "growth in the presence of sucrose" and "nongrowth in the presence of kanamycin" are investigated with
the aid of the polymerase chain reaction by the standard
PCR method of Innis et al. (PCR Protocols. A Guide to

30 Methods and Applications, 1990, Academic Press). A DNA
fragment which carries the lysE gene and surrounding
regions is amplified here from the chromosomal DNA of the
colonies. The following primer oligonucleotides are chosen
for the PCR.

lysek-1 (SEQ ID NO: 11): 5 TGC TTG CAC AAG GAC TTC AC 3

lysek-2 (SEQ ID NO: 12):
5 TAT GGT CCG CAA GCT CAA TG 3'

- The primers allow amplification of a DNA fragment approx.

  1.2 kb in size in control clones with the original lysE locus. In clones with a second copy of the lysC gene in the chromosome at the lysE locus, DNA fragments with a size of approx. 2.3 kb are amplified.
- The amplified DNA fragments are identified by means of electrophoresis in a 0.8% agarose gel. On the basis of the amplified fragment length, a distinction was made between clones with one chromosomal lysE gene copy and clones with two chromosomal lysE gene copies. It could thus be
- demonstrated that the strain ATCC21513\_17 carries two complete copies of the lysE gene on the chromosome.

The strain was deposited as C. glutamicum

The strain was called C. glutamicum ATCC21513\_17lysE::lysE.

ATCC21513\_17lysE::lysE on 5th June 2002 under number

20 DSM15037 at the Deutsche Sammlung für Mikroorganismen und
Zellkulturen (DSMZ, Braunschweig, Germany) in accordance
with the Budapest Treaty.

## Example 3

Generation of a tandem duplication of the zwal gene in the chromosome of Corynebacterium glutamicum

3.1. Construction of the tandem vector pK18mobsacBzwa1zwa1

Plasmid DNA was isolated from the Escherichia coli strain DSM13115 (EP-A-1111062), which carries the plasmid pCR2.1zwa1exp.

The plasmid contains the zwal gene which codes for cell growth factor 1. A pure culture of this strain was deposited on 19th October 1999 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) in accordance with the Budapest Treaty.

The plasmid pCR2.1zwalexp is cleaved with the restriction enzyme EcoRI (Amersham-Pharmacia, Freiburg, Germany), and after separation in an agarose gel (0.8%) the zwal fragment of 1 kb is isolated from the agarose gel with the aid of the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and employed for ligation with the mobilizable cloning vector pK18mobsacB described by Schäfer et al., Gene, 14, 69-73 (1994). This is cleaved beforehand with the restriction enzyme EcoRI and dephosphorylated with alkaline phosphatase (Alkaline Phosphatase, Boehringer Mannheim), mixed with the zwal fragment of 1 kb and the mixture is treated with T4 DNA Ligase (Amersham-Pharmacia, Freiburg, Germany).

The E. coli strain DH5α (Grant et al.; Proceedings of the National Academy of Sciences USA, 87 (1990) 4645-4649) is then transformed with the ligation batch (Hanahan, In. DNA Cloning. A Practical Approach. Vol. 1, ILR-Press, Cold Spring Harbor, New York, 1989). Selection of plasmid-carrying cells is made by plating out the transformation batch on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2<sup>nd</sup> Ed., Cold Spring Harbor, New York, 1989), which was supplemented with 25 mg/l kanamycin.

Plasmid DNA is isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by restriction cleavage with the enzyme NheI and subsequent agarose gel electrophoresis. Checking of the plasmid showed that two zwal fragments were cloned simultaneously and in the desired orientation in the cloning vector pK18mobsac.

The plasmid is called pK18mobsacBzwa1zwa1. A map of the plasmid is shown in Figure 3.

- 3.2. Generation of a tandem duplication of the zwa1 gene in C. glutamicum strain ATCC21513\_17
- The vector pK18mobsacBzwa1zwa1 mentioned in Example 3.1 is transferred by a modified protocol of Schäfer et al. (1990 Journal of Microbiology 172: 1663-1666) into the C. glutamicum strain ATCC21513\_17.
- The Corynebacterium glutamicum strain ATCC21513\_17 was produced by multiple, non-directed mutagenesis, selection and mutant selection from C. glutamicum ATCC21513. The strain is resistant to the lysine analogue S-(2-aminoethyl)-L-cysteine and both leucine- and homoserine-prototrophic.
- 15 The vector cannot replicate independently in ATCC21513\_17 and is retained in the cell only if it has integrated into the chromosome.

Selection of clones with integrated pK18mobsacBzwa1zwa1 is carried out by plating out the conjugation batch on LB agar 20 (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2<sup>nd</sup> Ed., Cold Spring Harbor, New York, 1989), which was supplemented with 15 mg/l kanamycin and 50 mg/l nalidixic acid. Clones which have grown on are plated out on LB agar plates with 25 mg/l kanamycin and incubated for 16 hours at 25 33°C. To achieve excision of the plasmid with only one copy of the zwal gene, the clones are cultured on LB agar with 10% sucrose, after incubation for 16 hours in LB liquid medium. The plasmid pK18mobsacB contains a copy of the sacB gene, which converts sucrose into levan sucrase, which is 30 toxic to C. glutamicum.

Only those clones in which the pK18mobsacBzwa1zwa1 integrated has been excised again therefore grow on LB agar with sucrose. Approximately 40 to 50 colonies are tested

for the PCR.

for the phenotype "growth in the presence of sucrose" and "non-growth in the presence of kanamycin". During the excision, either two copies of the zwal gene or only one can be excised together with the plasmid.

- To demonstrate that two copies of zwal have remained in the chromosome, approximately 20 colonies which show the phenotype "growth in the presence of sucrose" and "nongrowth in the presence of kanamycin" are investigated with the aid of the polymerase chain reaction by the standard PCR method of Innis et al. (PCR Protocols. A Guide to Methods and Applications, 1990, Academic Press). A DNA fragment which carries the zwal gene and surrounding regions is amplified here from the chromosomal DNA of the colonies. The following primer oligonucleotides are chosen
  - zwa1-A2 (SEQ ID NO: 13): 5` CAC TTG TCC TCA CCA CTT TC 3`

zwa1-E1 (SEQ ID NO: 14):
5` TTC TAC TGG GCG TAC TTT CG 3`

- The primers allow amplification of a DNA fragment approx.

  1.3 kb in size in control clones with the original zwal locus. In clones with a second copy of the zwal gene in the chromosome at the zwal locus, DNA fragments with a size of approx. 2.3 kb are amplified.
- The amplified DNA fragments are identified by means of electrophoresis in a 0.8% agarose gel. On the basis of the amplified fragment length, a distinction was made between clones with one chromosomal zwal gene copy and clones with two chromosomal zwal gene copies. It could thus be demonstrated that the strain ATCC21513\_17 carries two complete copies of the zwal gene on the chromosome.

The strain was called C. glutamicum ATCC21513\_17zwa1::zwa1. The strain was deposited as C. glutamicum

ATCC21513\_17zwa1::zwa1 on 5th June 2002 under number DSM15038 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) in accordance with the Budapest Treaty.

### 5 Example 4

### Preparation of Lysine

The C. glutamicum strains DSM13992lysC<sup>FBR</sup>::lysC<sup>FBR</sup>,
ATCC21513\_17lysE::lysE and ATCC21513\_17zwa1::zwa1 obtained
in Examples 1 to 3 are cultured in a nutrient medium
suitable for the production of lysine and the lysine
content in the culture supernatant was determined.

For this, the strains are first incubated on an agar plate for 24 hours at 33°C. Starting from this agar plate culture, a preculture is seeded (10 ml medium in a 100 ml conical flask). The medium MM is used as the medium for the preculture. The preculture is incubated for 24 hours at 33°C at 240 rpm on a shaking machine. A main culture is seeded from this preculture such that the initial OD (660 nm) of the main culture is 0.1 OD. The Medium MM is also used for the main culture.

5 g/l

### Medium MM

CSL

15

20

MOPS	20 g/l
Glucose (autoclaved separately)	50 g/l
Salts:	
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	25 g/l
KH <sub>2</sub> PO <sub>4</sub>	0.1 g/l
MgSO <sub>4</sub> * 7 H <sub>2</sub> O	1.0 g/l

CaCl <sub>2</sub> * 2 H <sub>2</sub> O	10 mg/l
FeSO <sub>4</sub> * 7 H <sub>2</sub> O	10 mg/1
MnSO <sub>4</sub> * H <sub>2</sub> O	5.0 mg/l
Biotin (sterile-filtered)	0.3 mg/l
Thiamine * HCl (sterile-filtered)	0.2 mg/l
CaCO <sub>3</sub>	25 g/l

The CSL (corn steep liquor), MOPS

(morpholinopropanesulfonic acid) and the salt solution are
brought to pH 7 with aqueous ammonia and autoclaved. The
sterile substrate and vitamin solutions, as well as the
5 CaCO<sub>3</sub> autoclaved in the dry state, are then added.

Culturing is carried out in a 10 ml volume in a 100 ml conical flask with baffles. Culturing is carried out at 33°C and 80% atmospheric humidity.

After 48 hours, the OD is determined at a measurement

10 wavelength of 660 nm with a Biomek 1000 (Beckmann

Instruments GmbH, Munich). The amount of lysine formed is

determined with an amino acid analyzer from Eppendorf
BioTronik (Hamburg, Germany) by ion exchange chromatography

and post-column derivation with ninhydrin detection.

15 The result of the experiment is shown in Table 10.

Table 10

Strain	OD (660 nm)	Lysine HCl g/l			
DSM13992	12.8	18.9			
DSM13992lysC <sup>FBR</sup> ::lysC <sup>FBR</sup>	12.0	21.6			
ATCC21513_17	10.4	14.0			
ATCC21513_17lysE::lysE	10.0	14.3			
ATCC21513_17zwa1::zwa1	9.9	14.6			

Brief Description of the Figures:

The base pair numbers stated are approximate values

5 obtained in the context of reproducibility of measurements.

Figure 1: Map of the plasmid pK18mobsacB2xlysCSma2/1.

The abbreviations and designations used have the following meaning:

KmR: Kanamycin resistance gene

HindIII: Cleavage site of the restriction enzyme

HindIII

BamHI: Cleavage site of the restriction enzyme

BamHI

lysC: lysC<sup>FBR</sup> allele lysC T311I

sacB: sacB gene

RP4mob: mob region with the replication origin for

the transfer (oriT)

oriV: Replication origin V

Figure 2: Map of the plasmid pK18mobsacB2xlysESma1/1.

The abbreviations and designations used have the following meaning:

KanR: Kanamycin resistance gene

SalI: Cleavage site of the restriction enzyme SalI

BamHI: Cleavage site of the restriction enzyme

BamHI

EcoRI: Cleavage site of the restriction enzyme

EcoRI

ScaI: Cleavage site of the restriction enzyme ScaI

lysE: lysE gene

sacB: sacB gene

RP4mob: mob region with the replication origin for

the transfer (oriT)

oriV: Replication origin V

Figure 3: Map of the plasmid pK18mobsacBzwa1zwa1.

5 The abbreviations and designations used have the following meaning:

KanR: Kanamycin resistance gene

ECORI: Cleavage site of the restriction enzyme

ECORI

NheI: Cleavage site of the restriction enzyme NheI

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zwa1: zwa1 gene

sacB: sacB gene

RP4mob: mob region with the replication origin for

the transfer (oriT)

oriV: Replication origin V

### INTERNATIONAL FORM

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Kantstr. 2

33790 Halle (Westf.)

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT issued pursuant to Rule 7.1 by the INTERNATIONAL DEPOSITARY AUTHORITY identified at the bottom of this page

L IDENTIFICATION OF THE MICROORGANISM					
Identification reference given by the DEPOSITOR: DSM13992lysCFBR::lysCFBR	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:  DSM 15036				
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESI	GNATION				
The microorganism identified under I. above was accompanied by:  (x) a scientific description (x) a proposed taxonomic designation  (Mark with a cross where applicable).					
III. RECEIPT AND ACCEPTANCE					
This International Depositary Authority accepts the microorganism identified under I. above, which was received by it on 2002-06-05 (Date of the original deposit).					
IV. RECEIPT OF REQUEST FOR CONVERSION					
The microorganism identified under I above was received by this International Depositary Authority on and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on for conversion).  (date of original deposit)					
V. INTERNATIONAL DEPOSITARY AUTHORITY					
Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH  Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):  Date: 2002-06-06				

Form DSMZ-BP/4 (sole page) 12/2001

Where Rule 6.4 (d) applies, such date is the date on which the status of international depositary authority was acquired.

### INTERNATIONAL FORM

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VIABILITY STATEMENT issued pursuant to Rule 10.2 by the INTERNATIONAL DEPOSITARY AUTHORITY identified at the bottom of this page

I. DEPOSITO	DR .	II. IDENTIFICATION OF THE MICROORGANISM
Name: Address:	Degussa AG Kantstr. 2 33790 Halle (Westf.)	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:  DSM 15036  Date of the deposit or the transfer!:  2002-06-05
II. VIABILI	TY STATEMENT .	
On that date,	of the microorganism identified under II above was tested on the said microorganism was	2002-06-05 2.
	Ono longer viable TIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PE	ERFORMED <sup>4</sup> .
v. intern	ATIONAL DEPOSITARY AUTHORITY	
Name:	DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):
Address:	Mascheroder Weg 1b D-38124 Braunschweig	Date: 2002-06-06

Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.

Mark with a cross the applicable box.

Fill in if the information has been requested and if the results of the test were negative.

### INTERNATIONAL FORM

Degussa AG

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VIABILITY STATEMENT issued pursuant to Rule 10.2 by the INTERNATIONAL DEPOSITARY AUTHORITY identified at the bottom of this page

DEPOSITO	OR .	II. IDENTIFICATION OF THE MICROORGANISM
ame: ddress:	Degussa AG Kantstr. 2 33790 Halle (Westf.)	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:  DSM 15037  Date of the deposit or the transfer!:  2002-06-05
il Viabili	TY STATEMENT	
n that date	of the microorganism identified under II above was tested on the said microorganism was  viable  no longer viable	2002-06-05
	TIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PER	RFORMED <sup>4</sup>
V. INTERN	ATIONAL DEPOSITARY AUTHORITY	
Name: Address:	DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):  Date: 2002-06-06

Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.

Mark with a cross the applicable box.

Fill in if the information has been requested and if the results of the test were negative.

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RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT issued pursuant to Rule 7.1 by the INTERNATIONAL DEPOSITARY AUTHORITY identified at the bottom of this page

L IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: ATCC21513_17lysE::lysE	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:  DSM 15037
IL SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC D	ESIGNATION
The microorganism identified under L above was accompanied by:  (X) a scientific description (X) a proposed taxonomic designation  (Mark with a cross where applicable).	
III. RECEIPT AND ACCEPTANCE  This International Depositary Authority accepts the microorganism identifit (Date of the original deposit).	ed under I. above, which was received by it on 2002-06-05
IV. RECEIPT OF REQUEST FOR CONVERSION  The microorganism identified under I above was received by this Internation and a request to convert the original deposit to a deposit under the Budapet for conversion).	onal Depositary Authority on (date of original deposit) at Treaty was received by it on (date of receipt of request
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH  Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):  Date: 2002-06-06

Where Rule 6.4 (d) applies, such date is the date on which the status of international depositary authority was acquired. Form DSMZ-BP/4 (sole page) 12/2001

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VIABILITY STATEMENT issued pursuant to Rule 10.2 by the INTERNATIONAL DEPOSITARY AUTHORITY identified at the bottom of this page

I. DEPOSIT	OR .	II. IDENTIFICATION OF THE MICROORGANISM				
Name: Address:	Degussa AG Kantstr. 2 33790 Halle (Westf.)	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:  DSM 15038  Date of the deposit or the transfer*:  2002-06-05				
III. VIABILI	TY STATEMENT.					
On that date,	the Salu Iniciourganism was	002-06-05				
	viable viable no longer viable					
IV. CONDIT	IONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFO	ORMED <sup>4</sup>				
v. interna	ATIONAL DEPOSITARY AUTHORITY					
Address:	DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):  Date: 2002-06-06				

Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer). In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.

Mark with a cross the applicable box.

Fill in if the information has been requested and if the results of the test were negative.

### INTERNATIONAL FORM

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RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT issued pursuant to Rule 7.1 by the INTERNATIONAL DEPOSITARY AUTHORITY identified at the bottom of this page

L IDENTIFIC	CATION OF THE MICROORGANISM					
Identification reference given by the DEPOSITOR:  ATCC21513_17zwa1::zwa1  Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:  DSM 15038						
II. SCIENTI	FIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	NATION .				
The microorganism identified under I. above was accompanied by:  (						
III. RECEIPT AND ACCEPTANCE  This International Depositary Authority accepts the microorganism identified under L above, which was received by it on 2002-06-05 (Date of the original deposit).						
IV. RECEIP	T OF REQUEST FOR CONVERSION					
The microorganism identified under I above was received by this International Depositary Authority on and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on for conversion).  (date of original deposit) (date of receipt of request						
V. INTERN	ATIONAL DEPOSITARY AUTHORITY					
Name: Address:	DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):  Date: 2002-06-06				

Where Rule 6.4 (d) applies, such date is the date on which the status of international depositary authority was acquired.

### INTERNATIONAL FORM

Degussa AG Kantstr. 2 33790 Halle/Künsebeck

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT issued pursuant to Rule 7.1 by the INTERNATIONAL DEPOSITARY AUTHORITY identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM					
Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:  DSM 14244					
GNATION					
This International Depositary Authority accepts the microorganism identified under I. above, which was received by it on 2001-04-20 (Date of the original deposit).					
The microorganism identified under I above was received by this International Depositary Authority on (date of original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion).					
V. INTERNATIONAL DEPOSITARY AUTHORITY					
Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):  Date: 2001-04-26					

Form DSMZ-BP/4 (sole page) 0196

Where Rule 6.4 (d) applies, such date is the date on which the status of International depositary authority was acquired.

#### INTERNATIONAL FORM

Degussa AG Kantstr. 2 33790 Halle/Künsebeck

VIABILITY STATEMENT issued pursuant to Rule 10.2 by the INTERNATIONAL DEPOSITARY AUTHORITY identified at the bottom of this page

. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM				
Name: Degussa AG Kantstr. 2 Address: 33790 Halle/Künsebeck	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 14244  Date of the deposit or the transfer!: 2001-04-20				
II. VIABILITY STATEMENT					
The viability of the microorganism identified under II above was tested on 2 On that date, the said microorganism was  (X) <sup>3</sup> viable  ( ) <sup>3</sup> no longer viable  IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PI					
V. INTERNATIONAL DEPOSITARY AUTHORITY					
Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH  Address: Mascheroder Weg 1b	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):				

Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

Mark with a cross the applicable box.

In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.

Fill in if the information has been requested and if the results of the test were negative.

### What is claimed is:

- Coryneform bacteria which produce chemical compounds, wherein instead of the singular copy of an open reading frame (ORF), gene or allele naturally present at the particular desired site (locus), these have at least two copies of the open reading frame (ORF), gene or allele in question, preferably in tandem arrangement, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable stransposition and no nucleotide sequence which imparts resistance to antibiotics being present at the particular site, and in that these optionally
- have at least a third copy of the open reading frame
  (ORF), gene or allele in question at a further gene
  site, no nucleotide sequence which is capable
  of/enables episomal replication in microorganisms, no
  nucleotide sequence which is capable of/enables
  transposition and no nucleotide sequence which imparts
  resistance to antibiotics being present at the further
  gene site.
  - 2. Coryneform bacteria according to claim 1 which produce chemical compounds, wherein the coryneform bacteria belong to the genus Corynebacterium.
    - Coryneform bacteria of the genus Corynebacterium according to claim 2 which produce chemical compounds, wherein these belong to the species Corynebacterium glutamicum.
- 30 4. Coryneform bacteria according to claim 1 which produce chemical compounds, wherein the chemical compound is a compound chosen from the group consisting of L-amino acids, vitamins, nucleosides and nucleotides.

5. Coryneform bacteria according to claim 1 which produce chemical compounds, wherein the chemical compound is one or more L-amino acids chosen from the group consisting of L-aspartic acid, L-asparagine, L-threonine, L-serine, L-glutamic acid, L-glutamine, glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan, L-proline and L-arginine.

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- 10 6. Coryneform bacteria according to claim 1 which produce chemical compounds, wherein the chemical compound is the amino acid L-lysine.
- 7. Coryneform bacteria which produce L-lysine, wherein instead of the singular copy of an open reading frame (ORF), gene or allele of lysine production naturally present at the particular desired site (locus), these have at least two copies of the open reading frame (ORF), gene or allele of lysine production in question, preferably in tandem arrangement, no nucleotide
- sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the particular site, and in that these

optionally have at least a third copy of the open reading frame (ORF), gene or allele of lysine production in question at a further gene site, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the further gene site.

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- 8. Coryneform bacteria according to claim 7 which produce L-lysine, wherein the coryneform bacteria belong to the genus Corynebacterium.
- Coryneform bacteria of the genus Corynebacterium
   according to claim 8 which produce L-lysine, wherein these belong to the species Corynebacterium glutamicum.
- 10. Coryneform bacteria according to claim 7 which produce L-lysine, wherein the copy of an open reading frame (ORF), gene or allele of lysine production is one or more of the open reading frames, genes or alleles chosen from the group consisting of accBC, accDA, cstA, cysD, cysE, cysH, cysK, cysN, cysQ, dapA, dapB, dapC, dapD, dapE, dapF, ddh, dps, eno, gap, gap2, gdh, gnd, lysC, lysCFBR, lysE, msiK, opcA, oxyR, ppc, ppcFBR, pgk, pknA, pknB, pknD, pknG, ppsA, ptsH, ptsI, ptsM, pyc, pyc P458S, sigC, sigD, sigE, sigH, sigM, tal, thyA, tkt, tpi, zwa1, zwf and zwf A213T.
- 11. Coryneform bacteria according to claim 7 which produce L-lysine, wherein the copy of an open reading frame
  20 (ORF), gene or allele of lysine production is one or more of the genes or alleles chosen from the group consisting of lysC<sup>FBR</sup> lysE and zwa1.
- 12. Coryneform bacteria according to claim 7 which produce L-lysine, wherein the copy of an open reading frame

  (ORF), gene or allele of lysine production is the lyse gene.
  - 13. Coryneform bacteria according to claim 7 which produce L-lysine, wherein the copy of an open reading frame (ORF), gene or allele of lysine production is the zwa1 gene.

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14. Coryneform bacteria according to claim 7 which produce L-lysine, wherein the copy of an open reading frame (ORF), gene or allele of lysine production is an lysCFBR

allele which codes for a feed back resistant form of aspartate kinase.

- 15. Coryneform bacteria according to claim 14 which produce L-lysine, wherein the feed back resistant form of aspartate kinase coded by the lysC<sup>FBR</sup> allele contains an amino acid sequence according to SEQ ID NO:2, SEQ ID NO:2 having one or more amino acid exchanges chosen from the group consisting of A279T, A279V, S301F, T308I, S301Y, G345D, R320G, T311I and S381F.
- 10 16. Coryneform bacteria according to claim 14 which produce L-lysine, wherein the feed back resistant form of aspartate kinase coded by the lysCFBR allele has an amino acid sequence according to SEQ ID NO:4.
- 17. Coryneform bacteria according to claim 14 which produce
  15 L-lysine, wherein the coding region of the lysCFBR
  allele has the nucleotide sequence of SEQ ID NO:3.
- 18. Coryneform bacteria according to claim 7 which produce L-lysine, wherein the further gene site is one or more of the sites chosen from the group consisting of aecD,
  20 ccpA1, ccpA2, citA, citB, citE, fda, gluA, gluB, gluC, gluD, luxR, luxS, lysR1, lysR2, lysR3, menE, mqo, pck, pgi and poxB.
- 19. Coryneform bacteria according to claim 7 which produce L-lysine, wherein the further gene site is one of more of the sites chosen from the group consisting of intergenic regions of the chromosome, prophages contained in the chromosome and defective phages contained in the chromosome.
- 20. Processes for the preparation of one or more chemical compounds, which comprise the following steps:
  - a) fermentation of coryneform bacteria, which

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- instead of the singular copy of an open reading frame (ORF), gene or allele naturally present at the particular desired site (locus), have at least two copies of the said open reading frame (ORF), gene or allele, preferably in tandem arrangement, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the particular site, and which
- ii) optionally have at least a third copy of the said open reading frame (ORF), gene or allele at a further gene site, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the further gene site,

under conditions which allow expression of the said open reading frames (ORFs), genes or alleles,

- b) concentration of the chemical compound(s) in the fermentation broth and/or in the cells of the bacteria,
- c) isolation of the chemical compound(s), optionally
- 30 d) with constituents from the fermentation broth and/or the biomass to the extent of > (greater than) 0 to 100%.

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- 21. Process according to claim 20, wherein the coryneform bacteria belong to the genus Corynebacterium.
- 22. Process according to claim 20, wherein the coryneform bacteria of the genus Corynebacterium belong to the species Corynebacterium glutamicum.

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- 23. Process according to claim 20, wherein the chemical compound is a compound chosen from the group consisting of L-amino acids, vitamins, nucleosides and nucleotides.
- 24. Process according to claim 20, wherein the chemical compound is one or more L-amino acids chosen from the group consisting of L-aspartic acid, L-asparagine, L-threonine, L-serine, L-glutamic acid, L-glutamine, glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan, L-proline and L-arginine.
  - 25. Process according to claim 20, wherein the chemical compound is L-lysine.
- 20 26. Process for the preparation of L-lysine, which comprises the following steps:
  - a) fermentation of coryneform bacteria, which
    - i) instead of the singular copy of an open reading frame (ORF), gene or allele of lysine production naturally present at the particular desired site (locus), have at least two copies of the said open reading frame (ORF), gene or allele, preferably in tandem arrangement, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which

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> imparts resistance to antibiotics being present at the particular site, and which optionally

ii) have at least a third copy of the said open reading frame (ORF), gene or allele of lysine production at a further gene site, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the further gene site,

under conditions which allow expression of the said open reading frames (ORFs), genes or alleles,

- b) concentration of the L-lysine in the fermentation broth and/or in the cells of the bacteria,
- c) isolation of the L-lysine, optionally

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- d) with constituents from the fermentation broth and/or the biomass to the extent of > (greater than) 0 to 100%.
  - 27. Process for the preparation of L-lysine according to claim 26, wherein the coryneform bacteria belong to the genus Corynebacterium.
- 25 28. Process for the preparation of L-lysine according to claim 26, wherein the coryneform bacteria of the species Corynebacterium belong to the species Corynebacterium glutamicum.
- 29.Process for the preparation of L-lysine according to
  30 claim 26, wherein the copy of an open reading frame
  (ORF), a gene or allele of lysine production is one or
  more of the open reading frames, genes or alleles

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chosen from the group consisting of accBC, accDA, cstA, cysD, cysE, cysH, cysK, cysN, cysQ, dapA, dapB, dapC, dapD, dapE, dapF, ddh, dps, eno, gap, gap2, gdh, gnd, lysC, lysC<sup>FBR</sup>, lysE, msiK, opcA, oxyR, ppc, ppc<sup>FBR</sup>, pgk, pknA, pknB, pknD, pknG, ppsA, ptsH, ptsI, ptsM, pyc, pyc P458S, sigC, sigD, sigE, sigH, sigM, tal, thyA, tkt, tpi, zwa1, zwf and zwf A213T.

- 30. Process for the preparation of L-lysine according to claim 26, wherein the copy of an open reading frame

  (ORF), gene or allele of lysine production is one or more of the genes or alleles chosen from the group consisting of lysCFBR, lysE and zwal.
  - 31. Process for the preparation of L-lysine according to claim 26, wherein the copy of an open reading frame (ORF), gene or allele of lysine production is the lyse gene.
    - 32. Process for the preparation of L-lysine according to claim 26, wherein the copy of an open reading frame (ORF), gene or allele of lysine production is the zwal gene.
    - 33. Process for the preparation of L-lysine according to claim 26, wherein the copy of an open reading frame (ORF), gene or allele of lysine production is the lysC<sup>PBR</sup> allele which codes for a feed back resistant form of aspartate kinase.
    - 34. Process for the preparation of L-lysine according to claim 33, wherein the feed back resistant form of aspartate kinase coded by the lysCFBR allele contains an amino acid sequence according to SEQ ID NO:2, SEQ ID NO:2 having one or more amino acid exchanges chosen from the group consisting of A279T, A279V, S301F, T308I, S301Y, G345D, R320G, T311I and S381F.

- 35. Process for the preparation of L-lysine according to claim 33, wherein the feed back resistant form of aspartate kinase coded by the lysCFBR allele has an amino acid sequence according to SEQ ID NO:4.
- 5 36.Process for the preparation of L-lysine according to claim 33, wherein the coding region of the lysCFBR allele has the nucleotide sequence of SEQ ID NO:3.

- 37. Process for the preparation of L-lysine according to claim 26, wherein the further gene site is one or more of the sites chosen from the group consisting of aecD, ccpA1, ccpA2, citA, citB, citE, fda, gluA, gluB, gluC, gluD, luxR, luxS, lysR1, lysR2, lysR3, menE, mqo, pck, pqi and poxB.
- 38. Process for the preparation of L-lysine according to

  claim 26, wherein the further gene site is one of more

  of the sites chosen from the group consisting of

  intergenic regions of the chromosome, prophages

  contained in the chromosome and defective phages

  contained in the chromosome.
- 39. Process for the production of coryneform bacteria which produce one or more chemical compounds, wherein
  - a) the nucleotide sequence of a desired ORF, gene or allele, optionally including the expression and/or regulation signals, is isolated,
- 25 b) at least two copies of the nucleotide sequence of the ORF, gene or allele are arranged in a row, preferably in tandem arrangement,
- c) the nucleotide sequence obtained according to b) is incorporated in a vector which does not replicate or replicates to only a limited extent in coryneform bacteria,

- d) the nucleotide sequence according to b) or c) is transferred into coryneform bacteria, and
- e) coryneform bacteria which have at least two copies of the desired ORF, gene or allele at the particular desired natural site instead of the singular copy of the ORF, gene or allele originally present are isolated, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics remaining at the particular natural site (locus), and optionally
- 15 f) at least a third copy of the open reading frame
  (ORF), gene or allele in question is introduced
  at a further gene site, no nucleotide sequence
  which is capable of/enables episomal replication
  in microorganisms, no nucleotide sequence which
  is capable of/enables transposition and no
  nucleotide sequence which imparts resistance to
  antibiotics remaining at the further gene site.
  - 40. The plasmid pK18mobsacB2xlysCSma2/1 shown in Figure 1 and deposited in the form of a pure culture of the strain E. coli DH50mcr/pK18mobsacB2xlysCSma2/1 (=DH5alphamcr/pK18mobsacB2xlysCSma2/1) under number DSM14244.

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- 41. The Corynebacterium glutamicum strain
  DSM13992lysCFBR::lysCFBR deposited in the form of a pure
  culture under number DSM15036.
- 42. The Corynebacterium glutamicum strain
  ATCC21513\_17lysE::lysE deposited in the form of a pure
  culture under number DSM15037.

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43. The Corynebacterium glutamicum strain
ATCC21513\_17zwa1::zwa1 deposited in the form of a pure
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Figure 1: Plasmid pK18mobsacB2xlysCSma2/1

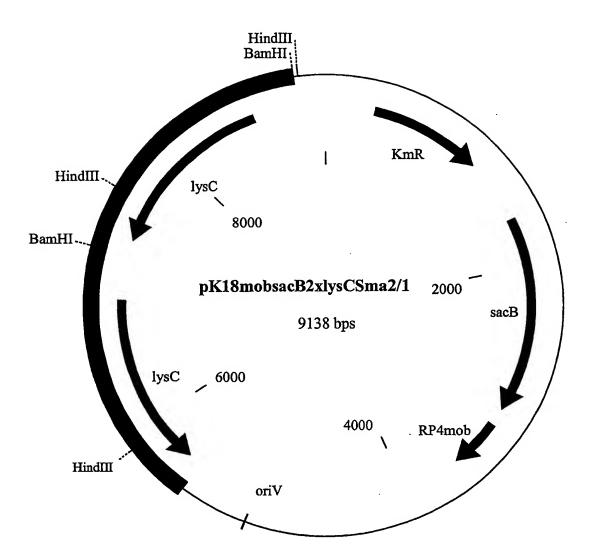


Figure 2: Plasmid pK18mobsacB2xlysESma1/1

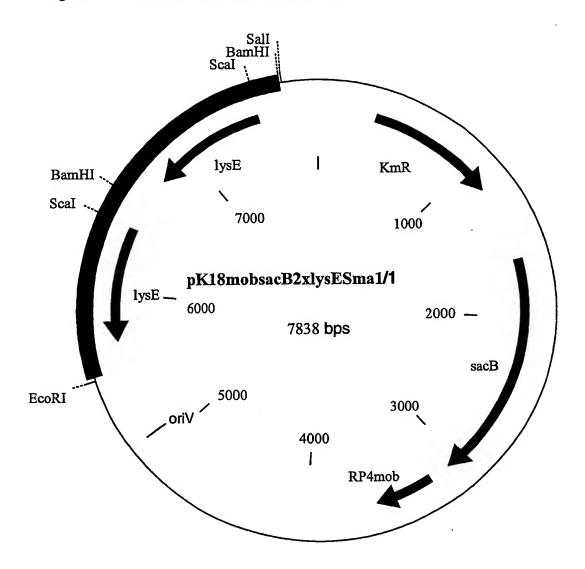
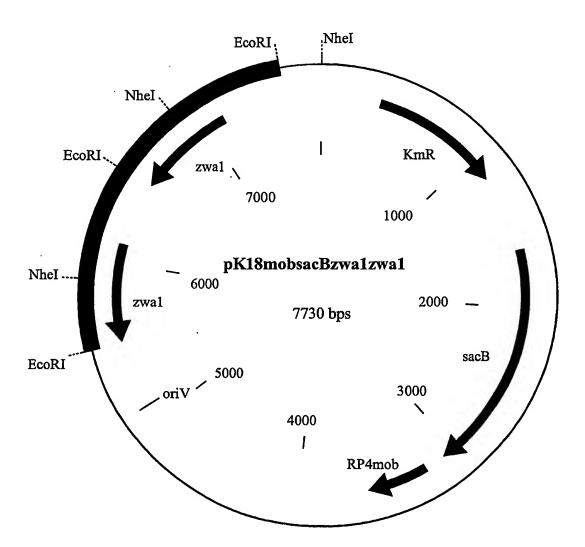


Figure 3: Plasmid pK18mobsacBzwa1zwa1



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- (71) Applicant (for all designated States except US): DE-GUSSA AG [DE/DE]; Bennigsenplatz 1, 40474 Düsseldorf (DE).
- (72) Inventors; and
- (75) Inventors/Applicants (for US only): Brigitte [DE/DE]; Twieten 1, 33154 Salzkotten (DE). KREUTZER, Caroline [DE/DE]; Poststrasse 16, 49326 Melle (DE). MÖCKEL, Bettina [DE/DE]; Benrodestrasse 35, 40597 Düsseldorf (DE). THIERBACH, Georg [DE/DE]; Gunststrasse 21, 33613 Bielefeld (DE).
- (74) Common Representative: DEGUSSA AG; Intellectual Property Management, Patents and Trademarks, Location Hanau, P. O. Box 13 45, 63403 Hanau (DE).
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### Declarations under Rule 4.17:

- as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii)) for the following designations AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZM, ZW, ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG)
- of inventorship (Rule 4.17(iv)) for US only

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with international search report

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: CORYNEFORM BACTERIA WHICH PRODUCE CHEMICAL COMPOUNDS II

(57) Abstract: The invention relates to coryneform bacteria, which instead of the singular copy of an open reading frame (ORF), gene or allele naturally present at the particular desired site (locus), have at least two copies of the open reading frame (ORF), gene or allele in question, preferably in tandem arrangement, and optionally at least a third copy of the open reading frame (ORF), gene or allele in question at a further gene site, and processes for the preparation of chemical compounds by fermentation of these bacteria.

PCT/EP 02/08465

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12P13/08 //C12R1/15

According to International Patent Classification (IPC) or to both national classification and IPC

### B. FIELDS SEARCHED

 $\begin{array}{ccc} \text{Minimum documentation searched (classification system followed by classification symbols)} \\ IPC & 7 & C12P & C12R \\ \end{array}$ 

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

WPI Data, PAJ, EPO-Internal, BIOSIS, MEDLINE

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 1 111 062 A (DEGUSSA) 27 June 2001 (2001-06-27) page 5, paragraph 30 -page 6, paragraph 35	1-43
X	EP 1 108 790 A (KYOWA HAKKO KOGYO KK) 20 June 2001 (2001-06-20) The copy only comprises the relevant parts of the document. page 24, paragraph 228; figure 1	1-43
<b>X</b>	US 6 200 785 B1 (KREUTZER CAROLINE ET AL) 13 March 2001 (2001-03-13) column 2, line 45 -column 4, line 6	1-43
X	WO 99 18228 A (PETERS WENDISCH PETRA; EIKMANNS BERND (DE); SAHM HERMANN (DE); KER) 15 April 1999 (1999-04-15) page 7, last paragraph	1-43

X Further documents are listed in the continuation of box C.	Patent family members are listed in annex.
Special categories of cited documents:  A' document defining the general state of the art which is not considered to be of particular relevance  E' earlier document but published on or after the International filing date  L' document which may throw doubts on priority ctalm(s) or which is cited to establish the publication date of another citation or other special reason (as specified)  O' document reterring to an oral disclosure, use, exhibition or other means  P' document published prior to the International filing date but later than the priority date ctalmed	"T" later document published after the International filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.  "&" document member of the same patent family
Date of the actual completion of the international search  10 June 2003	Date of mailing of the international search report  30/06/2003
Name and mailing address of the ISA  European Patent Office, P.B. 5818 Patentiaan 2  NL - 2280 HV Rijswijk  Tet. (+31-70) 340-2040, Tx. 31 651 epo nl,  Fax: (+31-70) 340-3016	Authorized officer  Griesinger, I

International Application No PCT/EP 02/08465

	kton) DOCUMENTS CONSIDERED TO BE RELEVANT  Citation of document, with Indication, where appropriate, of the relevant passages	Relevant to claim No.
	Citation of document, with Indication, where appropriate, of the relevant passages	Relevant to claim No.
Α		7
	SCHAEFER A ET AL: "SMALL MOBILIZABLE MULTI-PURPOSE CLONING VECTORS DERIVED FROM THE ESCHERICHIA COLI PLASMIDS PK18 AND PK19: SELECTION OF DEFINED DELETIONS IN THE CHROMOSOME OF CORYNEBACTERIUM GLUTAMICUM" GENE, ELSEVIER BIOMEDICAL PRESS. AMSTERDAM, NL, vol. 145, no. 145, 1994, pages 69-73, XP001093898 ISSN: 0378-1119 abstract	1-43
A	EIKMANNS B J ET AL: "MOLECULAR ASPECTS OF LYSINE, THREONINE, AND ISOLEUCINE BIOSYNTHESIS IN CORYNEBACTERIUM GLUTAMICUM"  ANTONIE VAN LEEUWENHOEK, DORDRECHT, NL, vol. 64, no. 2, 1993, pages 145-163, XP000918559  page 151, left-hand column, paragraph 1 page 157, left-hand column, paragraph 2	1-43
P, X	WO 02 22632 A (DEGUSSA) 21 March 2002 (2002-03-21) page 11, line 17 -page 15, line 13	1-43



Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)
This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:
Claims Nos.:     because they relate to subject matter not required to be searched by this Authority, namely:
2. X Claims Nos.: 1-43 (each claim partially) because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful international Search can be carried out, specifically:  see FURTHER INFORMATION sheet PCT/ISA/210
3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).
Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)
This International Searching Authority found multiple inventions in this international application, as follows:
As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
Remark on Protest  The additional search fees were accompanied by the applicant's protest.  No protest accompanied the payment of additional search fees.

### FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-43 (each claim partially)

The present set of claims refers to products and methods defined by 1. the absence of features, namely the absence of sequences which enable episomal replication and the absence of sequences enabling transposition and the absence of sequences providing antibiotics resistance and by presence of a vague feature, namely the presence of at least two copies of any kind of an ORF at a "particular desired site". The claims cover all products and methods having these characteristics, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such products and methods. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). The feature of an ORF "at a particular desired site" is completely unclear. The additional features are unclear, since they are defined as a result, which should not be achieved. This lack of clarity renders a meaningful search over the whole of the claimed scope impossible.

Furthermore, the present set of claims refers to any "chemical compound", which can be produced by Corynebacterium. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the compounds claimed, namely for lysine (see Table 1 and Examples 1-3) and in particular for the use of the genes lysC, lysE and zwal for the production of lysine. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible.

Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely 1. the principle of inserting two copies of a gene, which naturally occurs in Corynebacterium glutamicum, into the bacterial chromosome of Corynebacterium glutamicum wherein said two copies are only separated from each other by a "small" number of nucleotides, which do not encode a protein and wherein said bacterial chromosome does not contain any other vector sequences and 2. the specific embodiments relating to the insertion of two copies of the genes lysC, lysE or zwal as disclosed in the Examples 1-3. Said limitations of the search are based on the Examples 1-3.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

In nation on patent family members

International Application No PCT/EP 02/08465

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